

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 5/06</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/49136</b> <b>(43) International Publication Date:</b> 24 August 2000 (24.08.00)
<b>(21) International Application Number:</b> PCT/US00/03963 <b>(22) International Filing Date:</b> 16 February 2000 (16.02.00)  <b>(30) Priority Data:</b> 60/120,452 17 February 1999 (17.02.99) US  <b>(71) Applicant:</b> UNITED STATES SURGICAL [-/US]; 150 Glover Avenue, Norwalk, CT 06856 (US).  <b>(72) Inventors:</b> GRUSKIN, Elliott, A.; 23 Beach Tree Ridge, Killingworth, CT 06419 (US). TAWIL, Nabil; 844th 19th Street, Santa Monica, CA 90403 (US). GBIS, David; 77 Hibiscus Street #2, Fairfield, CT 06432 (US).  <b>(74) Agent:</b> FARBER, Mark; United States Surgical, 150 Glover Avenue, Norwalk, CT 06856 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> GENETICALLY ALTERED MESENCHYMAL STEM CELLS AND METHODS OF USE THEREOF		
<b>(57) Abstract</b>  Genetically altered mesenchymal stem cells include nucleic acid that encodes an immune system suppressor polypeptide which inhibits an immune response to the mesenchymal stem cells in a recipient of the mesenchymal stem cells. Such cells are useful in treating or correcting tissue injury and/or tissue disorders such as connective tissue disorders. The immune system suppressor polypeptide includes complement restriction factors, and enzymes or proteins which, when expressed in a xenogeneic cell, cause the reduction of a xenogeneic epitope on the cell-surface of the xenogeneic cell.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**GENETICALLY ALTERED MESENCHYMAL STEM CELLS**  
**AND METHODS OF USE THEREOF**

5

**BACKGROUND****Technical Field**

Genetically altered mesenchymal stem cells which express polypeptides involved in suppression of an immune response in a recipient receiving such stem cells, the production of such cells, and their use in the treatment of tissue injuries and/or tissue disorders.

10

**Description of Related Art**

Mesenchymal stem cells originate from mesodermal tissue such as bone marrow, blood, dermis, and periosteum. These cells are the progenitors for various types of structural and connective tissue including cartilage, bone, muscle, tendon, ligament, bone marrow stroma, and dermis.

15

The isolation and cultural-expansion of human mesenchymal stem cells has been described, e.g., in Caplan et al., U.S. Patent 5,486,359. In addition, genetically engineered human mesenchymal stem cells which produce proteins not normally produced in such cells have been described, e.g., in Gerson et al., U.S. Patent 5,591,625.

20

The ability of mesenchymal stem cells to differentiate into various tissue types has prompted investigators to apply mesenchymal stem cells to situations where such differentiation may provide therapeutic modalities to treat or repair tissue defects. One approach to treating patients suffering from connective tissue disorders has been the use of isolated and expanded human mesenchymal stem cells as described, e.g., in Caplan et al., U.S. Patent 5,226,914. These cells are isolated from the bone marrow of patients in need of such treatment, grown in culture, and then reintroduced into the same patient to correct the disorder. Alternatively, the mesenchymal stem cells may be obtained from a

25

human donor as in the case of allogeneic transplantation (transplantation of an organ from a donor species to a recipient of the same species).

While allogeneic transplantation of a variety of organs, tissues and cells, in general has been successful, a major disadvantage in using allogeneic transplantation is the shortage of human donors due to the increasing demand for this type of treatment, which cannot be met by existing sources. In addition, allogeneic transplants may be rejected from the transplant host by an immune response.

One approach to compensate for the increasing demand for organs, tissues, and cells is xenotransplantation which is defined as the transplantation of organs, tissues, and cells between animals of different species. Xenotransplantation can be classified as either concordant or discordant. Concordant refers to transplantation between closely related species, such as from baboon or chimpanzee to human. Discordant refers to transplantation between distantly related species, such as from pig to human. Although the transplantation of organs from non-human primates to humans has shown some success, the use of primates as donors of organs or tissues has not been feasible due to supply and ethical considerations, and concerns regarding the potential transmission of viral infections. Accordingly, clinical research has focused on the use of non-primate species, particularly from discordant species as a source of donor organs and tissues.

Particular attention has focused on the use of pigs as suitable donors in xenotransplantation. See, e.g., Cooper et al., "The pig potential organ donor for man", pp. 481-500, Cooper, D.K.C., Kemp, E., Reemtsma, K., and White, D.J.G. (eds), *Xenotransplantation*, Springer-Verlag, Berlin, Heidelberg, New York, 1991. However, discordant xenotransplantation such as from pig to human is typically followed by hyperacute rejection of the transplanted organ or tissue which is mediated by the action of natural antibodies and the activation of the complement cascade pathway. See, e.g., Dalmaso et al., "The complement system in xenotransplantation", *Immunopharmacology* 24: 149-160, 1992.

The complement system is well known, and is described, e.g., in Paul, W.E., *Fundamental Immunology*, pp. 19-20 and 931-939, Third Edition, Raven Press,

New York, 1993; and Makrides, "Therapeutic Inhibition of the Complement System", *Pharmacological Reviews* 50: 59-87, 1998, both of which are herein incorporated by reference. The system comprises more than 30 proteins which act together in a cascade sequence to cause destruction of invading microorganisms. During the course of

5 complement activation, proinflammatory peptides such as the anaphylatoxins, C3a and C5a, are produced and the membrane attack complex, C5b-9, is formed. The products formed by complement activation, particularly the anaphylatoxins, evoke a number of biological effects such as degranulation of phagocytic cells, mast cells and basophils, and chemotaxis of leukocytes.

10 Complement can be activated by two pathways, the classical and the alternative pathway. The classical pathway is activated when a complex of IgM or IgG antibodies binds to the first component of the classical pathway C1, which subsequently activates C4 and C2, which in turn, combine in a enzymatic complex to form a C3

15 convertase. This enzyme cleaves C3 to produce C3a and C3b which have distinct functions. C3a promotes local inflammatory activity, and is no longer involved in the complement pathway. C3b binds to C3 convertase to yield C5 convertase. Subsequently, this enzyme cleaves C5 into C5a and C5b, the latter of which binds to C6, C7 and C8 to form the complex C5b-8, which in turn, catalyzes the polymerization of C9, to form the membrane attack complex (MAC). This complex inserts into target cell membranes,

20 thereby causing cell lysis.

The alternative pathway is activated by microbial surfaces and a number of complex polysaccharides. C3b, which is provided by the spontaneous proteolysis of C3, forms a complex with factor B. The resulting complex is subsequently split by factor D to generate C3bBb, the convertase of the alternative pathway, which as in the classical

25 pathway cleaves bound C5, thereby initiating assembly of the membrane attack complex as described above.

Complement activation in an individual is ordinarily controlled by complement restriction factors present in the plasma or expressed on the cell surface. The C3 and C5 convertases of the classical pathway are regulated by members of the

Regulators of Complement Activation (RCA) family which include the cell-surface proteins complement receptor 1 (CR1; C3b/C4b receptor; CD35), complement receptor type 2 (CR2; CD21; Epstein-Barr virus receptor), membrane cofactor protein (MCP; CD46; measles virus receptor), decay-accelerating factor (DAF; CD55), and the plasma proteins factor H and C4b-binding protein (C4-bp). The C3 and C5 convertases of the alternative pathway are regulated by CR1, DAF, MCP and factor H. Proteins involved in the regulation of MAC are the plasma proteins, vitronectin (S-protein) and clusterin (SP-40,40), and the cell-surface proteins, CD59, and homologous restriction factor (HRF; C8bp).

These complement restriction factors are usually species-specific, and thus function effectively to inhibit complement activation only with the complement proteins of their own species (Dalmasso et al., "Inhibition of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor", *Transplantation* 52: 530-33, 1991).

Certain strategies have been developed to inhibit hyperacute rejection in xenotransplantation. For example, one strategy focuses on blocking complement activation through the use of complement inhibitors such as cobra venom factor, FUT-175 and K76COOH as described, e.g., in Hayashi et al., "Evidence that combination therapy using cobra venom factor, splenectomy, and deoxyspregualin is effective in guinea pig to rat cardiac xenotransplantation", *Transplantation* 57: 777-779, 1993; and Kobayashi et al., "Investigation of the FUT-175 and K76COOH, in discordant xenotransplantation", *Xenotransplantation* 3: 237-245, 1996. Another strategy has aimed to block the interactions between natural xenoreactive antibodies and xenoantigens as described, e.g., in Makrides, "Therapeutic Inhibition of the Complement System", *Pharmacological Reviews* 50: 59-87, 1998.

Other investigators have focused on the application of genetic engineering for xenotransplantation by transferring complement restriction factor genes onto the surface of xenogeneic cells. For example, insertion of human DAF into porcine endothelial cell membranes by its glycosphosphatidyl inositol anchor prevented lysis of these cells by human complement. See, e.g., (Dalmasso et al., *Transplantation* 52: 530-

533, *supra*. Further, transfection of the complement regulatory genes of human MCP, DAF and CD59 into xenogeneic cells was shown to inhibit complement-mediated cytotoxicity (Hayashi, "Xenotransplantation with Special Reference to Genetic Engineering", *Microbiol. Immunol.* 41: 751-756, 1997).

5                   This research has been followed by the production of transgenic animals which expressed human complement regulatory proteins on their tissue. See, e.g., Langford et al., "Production of pigs transgenic for human decay accelerating factor", *Transplant. Proc.* 26: 1400-1401, 1994; Cary et al., "Tissue expression of human decay accelerating factor, a regulator of complement activation expressed in mice: A potential  
10 approach to inhibition of hyperacute xenograft rejection", *Transplant. Proc.* 25: 400-401 (1993); Diamond et al., "Cell and tissue specific expression of a human CD59 minogene in transgenic mice", *Transplant. Proc.* 26: 1239, 1994; Rosengard et al., "Tissue Expression of human complement inhibitor, decay-accelerating factor, in transgenic pigs-a potential approach for preventing xenograft rejection", *Transplantation*  
15 59: 1325-1333, 1995; and Fodor et al., "Expression of a functional human complement inhibitor in transgenic swine as an approach to prevent xenogeneic hyperacute organ rejection", *Proc. Natl. Acad. Sci. U.S.A.* 91: 11153-11157, 1994.

                  Other studies on preventing or reducing hyperacute rejection of xenotransplants have focused on reducing expression of xenogeneic antigens which bind  
20 to preformed natural human antibodies. For example, pig epithelium expresses a Gal alpha(1,3)Gal epitope, which is responsible for binding of a large number of human natural antibodies (Oriol et al., "Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man xenotransplantation", *Transplantation* 56: 1433-42, 1995). This epitope is formed by the  
25 action of alpha-galactosyl transferase, which glycosylates N-acetyllactosamine. Human and old-world primates lack this enzyme, which in turn, results in the lack of the alpha-galactosyl epitope. As a consequence, human and old-world primates produce anti-galactosyl antibodies which occur from continuous exposure to the antigen on bacteria and gut flora. One strategy that has been effective in reducing expression of the alpha-

galactosyl epitope has been the transduction of the human enzyme, alpha-1,2 fucosyltransferase into animal cells, which utilizes N-acetyllactosamine in the same way as alpha-galactosyltransferase, to yield H substance. See, e.g., Koike et al., "Introduction of alpha (1,2)-fucosyltransferase and its effect on alpha-Gal epitopes in transgenic pig", *Xenotransplantation* 3: 81-86, 1996. Other studies of animals cells expressing the human enzyme lysosomal alpha-galactosidase have also shown a reduction of the epitope Gal alpha(1,3) Gal, a reduction in the binding of natural human antibodies to this epitope, and inhibition of human antibody-mediated lysis. See, e.g., Osman et al., "Combined transgenic expression of alpha-galactosidase and alpha 1,2 fucosyltransferase leads to optimal reduction in the major xenoepitope Gal alpha(1,3) Gal", *Proc. Natl. Acad. Sci. USA* 94: 14677-14682, 1997.

With respect to the treatment of patients suffering from tissue injuries or tissue disorders, and in view of the shortage of human donors, there is a need to find an alternative source of non-immunogenic donor mesenchymal stem cells for use in transplantation.

## **SUMMARY**

In one aspect, the embodiments described herein address the problems discussed above by providing genetically altered mesenchymal stem cells that, when transplanted into a recipient do not trigger an immune response and are not rejected by the recipient. The altered mesenchymal stem cells are useful in the treatment of tissue disorders and tissue injuries, such as those occurring in connective tissue.

An isolated mesenchymal stem cell is provided which includes nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response to the mesenchymal stem cell in the recipient.

A composition is also provided which includes an isolated mesenchymal stem cell having nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response to the



mesenchymal stem cell in the recipient, and a culture medium for growth, which allows the reproduction of the mesenchymal stem cell.

5 A therapeutic composition for treating tissue in need of repair or reconstruction is also provided which includes an isolated mesenchymal stem cell having nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response to the mesenchymal stem cell in the recipient, and a pharmaceutically acceptable carrier, the mesenchymal stem cell being present in an amount effective to aid in repair or reconstruction of the tissue by differentiating into a cell which is normally indigenous to the tissue. In one aspect the mesenchymal stem cell differentiates into a cell which is normally indigenous to tissue selected from the group consisting of bone, cartilage, muscle, tendon and ligament.

10 A method of treating tissue in need of repair or reconstruction is also provided which includes administering to a subject having such tissue, an effective repair or reconstructing amount of a therapeutic composition including an isolated mesenchymal stem cell including nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response to the mesenchymal stem cell in the recipient, and a pharmaceutical acceptable carrier, sufficient to treat the tissue.

20 A method of obtaining a genetically altered mesenchymal stem cell which does not trigger an acute immune response in a recipient of the mesenchymal stem cell is also provided which includes isolating a mesenchymal stem cell, introducing a vector including nucleic acid which encodes an immune system suppressor polypeptide into the isolated mesenchymal stem cell, to provide the genetically altered mesenchymal stem cell.

25 A method of obtaining a genetically altered mesenchymal stem cell which does not trigger an acute immune response in a recipient of the mesenchymal stem cell is also provided which includes obtaining a transgenic animal which has been engineered to produce cells having an immune system suppressor polypeptide, the immune system

suppressor polypeptide adapted to reduce or inhibit an immune response to cells from the transgenic animal to the recipient, and isolating mesenchymal stem cells from the transgenic animal.

## 5 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a photomicrograph of a monolayer culture of undifferentiated mesenchymal stem cells derived from porcine bone marrow.

Figure 2 is a graph of absorbance at wavelength 405nm versus cell number. The graph shows the differentiation of genetically altered mesenchymal stem  
10 cells into osteoblasts as measured through the production of alkaline phosphatase in the medium.

Figure 3 is a graph of cell number versus fluorescence intensity. The graph shows the results of fluorescence activated cell sorter (FACS) analysis of the cell surface expression of CD59 mesenchymal stem cells isolated from transgenic pigs  
15 expressing CD59 relative to mesenchymal stem cells isolated from non-transgenic pigs.

Figures 4A and 4B are graphs showing protection of transgenic porcine mesenchymal stem cells expressing CD59 from lysis by human serum complement, dye release (%) versus human serum (%) for non-transgenic porcine mesenchymal stem cells (open diamonds), CD59-transgenic porcine mesenchymal stem cells (crosses), and 3T3  
20 fibroblasts (pluses). Figure 4A shows protection of transgenic mesenchymal stem cells at low cell density, where Figure 4B shows protection of transgenic mesenchymal stem cells at high cell density.

Figures 5A and 5B are photomicrographs showing positive results from the indirect immunofluorescence analysis of cultured porcine mesenchymal stem cells  
25 transfected with human H-transferase. Figure 5A shows the expression of human H-transferase on porcine mesenchymal stem cells transfected with vector containing the human H-transferase gene. The transfected mesenchymal stem cells are labeled with human H-transferase marker (UEA-1). Figure 5B represents a negative control of porcine mesenchymal stem cells transfected with vector alone.

Figure 6 is a graph of cell number versus fluorescence intensity. The graph shows the results of FACS analysis of the cell surface expression of human H-transferase on porcine mesenchymal stem cells transfected with vector carrying the human H-transferase gene relative to porcine mesenchymal stem cells transfected with vector alone.

Figure 7 is a graph of cell number versus fluorescence intensity. The graph shows the results of FACS analysis of the cell surface expression of human H-transferase on porcine mesenchymal stem cells transfected with vector carrying the human H-transferase gene before and after two rounds of sorting.

Figure 8 is a graph of cell number versus fluorescence intensity. The graph shows the results of FACS analysis of the cell-surface expression of human H-transferase on porcine mesenchymal stem cells transfected with vector carrying the human H-transferase gene relative to porcine mesenchymal stem cells transfected with vector alone, and non-transfected rabbit and monkey mesenchymal stem cells.

Figure 9 is a graph of cell number versus fluorescence intensity. The graph shows the results of FACS analysis of the cell surface expression of the Gal alpha(1,3) Gal epitope in porcine mesenchymal stem cells transfected with vector carrying the human H-transferase gene relative to porcine mesenchymal stem cells transfected with vector alone, and non-transfected rabbit and monkey mesenchymal stem cells.

Figures 10A and 10B are photomicrographs of the growth of porcine mesenchymal cells on melted mesh composed of an 18/82 (mole percent) glycolide-lactide copolymer at 2 hours (A) and 3 days (B).

Figures 11A and 11B are photomicrographs (10x and 40x, respectively) of the growth of porcine mesenchymal stem cells on mesh composed of an 18/82 (mole percent) glycolide-lactide copolymer after 8 days.

Figures 12A and 12B are photomicrographs (100x) of porcine mesenchymal stem cells grown on mesh for 3 days. Figure 12A shows mesh without mesenchymal stem cells. Figure 12B shows the growth of mesenchymal cells on mesh.

**DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

Mesenchymal stem cells which are genetically altered in accordance with the disclosure herein to express an immune system suppressor polypeptide reduce or inhibit hyperacute rejection of the mesenchymal stem cells when they are transplanted in a recipient by inhibiting an immune response in that recipient. An "immune response" herein refers to the reaction of a recipient of mesenchymal stem cells in response to an antigen, and involves antibody production and/or cell-mediated immunity.

The genetically altered mesenchymal stem cells disclosed herein are not rejected when transplanted into a suitable recipient, particularly a discordant species, as is the case with unaltered xenogeneic mesenchymal stem cells. Accordingly, genetically altered mesenchymal stem cells herein overcome a major problem attending xenotransplantation, i.e. hyperacute rejection, and thus, provide a solution to the problem of a shortage of allogeneic donor cells. The altered mesenchymal stem cells differentiate into specific types of tissue in the presence of suitable stimulating agents, and thus are useful in the treatment of tissue disorders and tissue injuries, e.g., the regeneration and repair of bone and/or cartilage defects.

In one embodiment, genetically altered mesenchymal stem cells are expanded in a suitable medium and transplanted into a recipient at a desired locus where they differentiate into the desired tissue. Since the differentiation occurs within the tissue being treated, the cells are incorporated directly into the naturally occurring repair and reconstruction process. In this manner, the natural healing process is bolstered, or in the case of pathological conditions, deficiencies which lead to failure of natural repair and reconstruction are corrected.

Genetically altered mesenchymal stem cells herein can be obtained by two different approaches. In one aspect, previously isolated mesenchymal stem cells from normal (non-transgenic) animals are transfected with nucleic acid coding for an immune system suppressor polypeptide which is expressed by the mesenchymal stem cell. In another aspect, genetically altered mesenchymal stem cells are obtained from transgenic animals which have incorporated into their genome nucleic acid coding for an immune

system suppressor polypeptide, which is expressed by the mesenchymal stem cells. The stem cells are then isolated, and optionally grown to yield greater numbers of such cells.

Suitable mesenchymal stem cells can be obtained from any animal, preferably from a mammal. Suitable mammals include, but are not limited to, pig, mouse, sheep, cow, goat, rabbit, and the like. Sources of suitable mesenchymal stem cells are, e.g., bone marrow, blood, periosteum, dermis, and other tissues of mesodermal origin. One preferred embodiment contemplates the isolation of mesenchymal stem cells from bone marrow, which contains the highest percentage of mesenchymal stem cells compared with other sources of these cells.

Methods of obtaining mesenchymal stem cells from natural sources are well-known. It is contemplated that any method for obtaining mesenchymal stem cells from natural sources which are known to those with skill in the art may be utilized. A more detailed discussion of methods of obtaining mesenchymal stem cells for animals is given below.

In one aspect, previously isolated naturally occurring mesenchymal stem cells are genetically altered to express an immune system suppressor polypeptide by using conventional *in vitro* transfection techniques. Suitable vectors for introducing DNA encoding the immune system suppressor polypeptide into the isolated mesenchymal stem cells are well-known and include, but are not limited to, a retroviral vector, or other viruses such as SV40, herpes virus, adeno virus, human papilloma virus, or bacterial plasmids as described, e.g., in Gerson et al., U.S. Patent 5,591,625, Fodor et al., U.S. Patents 5,624,837 and 5,627,264, and Sims et al., U.S. Patent 5,573,940, all of which are herein incorporated by reference.

Examples of suitable promoters and enhancers used to direct expression in mesenchymal stem cells are well-known in the art as described, e.g., in Gerson et al., U.S. Patent 5,591,625, Fodor et al., U.S. Patents 5,624,837 and 5,627,264 and Sims et al., U.S. Patent 5,573,940.

The expression vectors may preferably contain selection genes. Generally, the selection genes utilized are those encoding proteins that confer resistance upon a host

cell, as for example, the beta lactamase antibiotic resistance gene which confers resistance to cells in the presence of the antibiotic, ampicillin, and the neomycin gene which confers resistance to cells in the presence of the cytotoxic aminoglycoside, G418 or others such as dihydrofolate reductase.

5                   Methods of introducing the vector carrying nucleic acid encoding for the immune system suppressor polypeptide include, but are not limited to, microinjection, electroporation, transfection using DEAE dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art. See Sambrook et al., in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New  
10                  York, 1989, herein incorporated by reference.

                  The immune system suppressor polypeptide encoded by the nucleic acid introduced into isolated mesenchymal stem cells refers to a polypeptide that, when expressed by an isolated mesenchymal stem cell transplanted into a recipient, functions to avoid, inhibit or otherwise reduce an immune response in the recipient of the transplanted  
15                  stem cell. Such a polypeptide includes, but is not limited to, complement restriction factors, and enzymes or proteins, such as glycosyltransferases that when expressed by mesenchymal stem cells, cause a reduction in the expression of a xenogeneic antigen normally present on the surface of the donor mesenchymal stem cell.

                  Complement restriction factors refer to substances that regulate  
20                  complement-mediated activation, thereby preventing cytolysis. These factors permit complement to distinguish "self" from "non-self" (foreign particles) and thus prevent complement activation on autologous tissue. Since these factors are species-specific, the complement restriction factors present in donor tissue of a different species from that of the recipient, particularly discordant species, are not able to regulate complement  
25                  activation of the recipient, thereby resulting in hyperacute rejection of the transplanted donor tissue. Incorporation of a recipient's complement restriction factor(s) in a non-native mesenchymal stem cell thus camouflages the altered mesenchymal stem cell and prevents generation of the complement cascade.

Suitable complement restriction factors are either cell-surface proteins which include, but are not limited to, DAF, MCP, CD59, CR1, CR2, and HRF; or restriction factors present in the serum which include, but are not limited to, factor H, C1 inhibitor, C4-bp, S-protein, SP-40, 40 and combinations thereof. The structure and function of these restriction factors are reviewed in Makrides, "Therapeutic Inhibition of the Complement System", *Pharmacological Reviews*, 50: 59-87, 1998, herein incorporated by reference. Preferably, the complement restriction factor is a cell-surface protein and includes, but is not limited to, DAF, MCP, CD59, CR1, CR2, HRF and combinations thereof. More preferred cell-surface complement restriction factors include DAF, CD59, MCP and combinations thereof. A brief summary of the more preferred cell-surface complement restriction factors is set forth below.

DAF (also referred to as CD55)- is made of up four short consensus repeats and a serine/threonine domain that is capable of extensive O-linked glycosylation. It is attached to the cell membrane by a glycosyl phosphatidyl inositol anchor, and accelerates the decay of C3/C5 convertases. The nucleotide and amino acid sequences for human DAF are described, e.g., in Fodor et al., U.S. Patent No. 5,627,264, herein incorporated by reference.

MCP (also referred to as CD46)- is a membrane glycoprotein that has factor I cofactor activity, and is expressed as four isoforms referred to as BC1, BC2, C1 and C2. It acts together with DAF to block the deposit of C3b/C4b on cell membranes. The nucleotide and amino acid sequences for human MCP are described in Fodor et al., U.S. Patent No. 5,627,264.

CD59- is a single chain glycoprotein that is anchored to the membrane through a glycosyl phosphatidyl inositol anchor. This protein inhibits complement activation by binding to C8 and C9, which blocks the addition of polymerized C9 molecules. The nucleotide and

amino acid sequences for human CD59 are described, e.g., in Sims et al., U.S. Patent No. 5,573,940, herein incorporated by reference.

Suitable complement restriction factors also include hybrids of the above-mentioned complement restriction factors. Chimeric proteins such as DAF/CD59 have  
5 been constructed and have been shown to exhibit both DAF and CD59 activity as described, e.g., in Foder et al., "A novel bifunctional chimeric complement inhibitor that regulates C3 convertase and formation of the membrane attack complex", *J. Immunol.* 155: 4135-4138, 1995. Chimeric proteins of MCP and DAF have also been shown to possess both factor I cofactor activity and decay-accelerating activity as described, e.g., in  
10 Miyagawa et al., "Effect of transfectant molecules, MCP, DAF and MCP/DAF hybrid on xenogeneic vascular endothelium", *Transplant Proc.* 26: 1253-1254, 1994. Chimeric proteins which block complement activation are also described in Ko et al., U.S. Patent No. 5,679,546; Fodor et al., U.S. Patent 5,624,837; and Fodor et al., U.S. Patent 5,627,264, all of which are herein incorporated by reference. Moreover, suitable  
15 complement restriction factors include analogs thereof as described in Atkinson et al., U.S. Patent 5,719,127, herein incorporated by reference.

Suitable proteins or enzymes that when expressed in xenogeneic cells are capable of reducing the expression of xenogeneic antigens, and thus reduce or inhibit rejection of the xenogeneic cell when transplanted in a recipient include, but are not  
20 limited to, glycosyltransferases such as human alpha-1,2 fucosyltransferase (H-transferase) and human lysosomal alpha-galactosidase. Transfection of these genes into porcine endothelial cells, COS cells and other cells has been described, e.g., in Osman et al., "Combined transgenic expression of alpha-galactosidase and alpha 1,2-fucosyltransferase leads to optimal reduction in the major xenoepitope Gal  
25 alpha(1,3)Gal", *Proc. Natl. Acad. Sci. USA* 94: 14677-14682, 1997; herein incorporated by reference. The nucleotide and amino acid sequences for human H-transferase is set forth as SEQ ID NO: 1 and SEQ ID NO: 2, respectively.



The particular nucleic acid sequences encoding immune system suppressor polypeptides have been identified as described above, and may be isolated by techniques that are well known to those skilled in the art as described, e.g., in Sambrook et al., *supra*.

5 In another aspect, genetically altered mesenchymal stem cells may be obtained by producing transgenic animals that express an immune system suppressor polypeptide, followed by isolation of the mesenchymal stem cells from the transgenic animal. Methods of isolating mesenchymal stem cells from such transgenic animals are the same as those involved in obtaining mesenchymal stem cells from naturally occurring animals, and are described below. Preferably, transgenic animals used herein provide  
10 germ-line transmission of the trait for producing immune system suppressor polypeptides.

Methods for producing transgenic animals are well known in the art and include, but are not limited to, microinjection, e.g., of pronuclei, electroporation of ova or zygotes, nuclear transplantation, and/or the stable transfection or transduction of embryonic stem cells obtained from the donor animal as described, e.g., in Fodor, U.S.  
15 Patent 5,624,837.

A common element of these methods involves the preparation of a transgene transcription unit as described, e.g., in Fodor et al., U.S. Patent No. 5,624,837, which comprises DNA molecules which usually include 1) a promoter, 2) the nucleic acid sequence of interest, i.e. the sequence encoding the immune system suppressor  
20 polypeptide, and 3) a polyadenylation signal sequence. Other sequences, such as enhancer and intron sequences, may be included. The unit is prepared by isolating a restriction fragment of a plasmid vector which expresses the immune system suppressor polypeptide in, e.g., mammalian cells. Preferably, the restriction fragment is free of sequences which direct replication in bacterial host cells since these sequences may  
25 detrimentally affect embryo viability.

One successful well-known method of producing transgenic animals, particularly large mammals, e.g., pig, cow, horse, rabbit, etc., involves the introduction of at least one clone of isolated nucleic acid molecules encoding the protein of interest, i.e., an immune system suppressor polypeptide, into at least one blastomere of a multi-cellular

embryo, i.e., having at least three cells, as described, e.g., in Ebert', et al., "Changes in Domestic Ungulates through Genetic Engineering" in *Animal Applications in Mammalian Development*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1991; and Ebert et al., *Animal Biotechnology* 1:145-159, 1990, both of which are  
5 incorporated herein by reference. The method involves harvesting embryos from a donor female and introducing isolated nucleic acid molecules into a blastomere having a paternal genome (the portion of the embryo's genome contributed by the sperm) before transcription of the paternal genome of the embryo occurs.

Another method of producing transgenic animals, particularly transgenic  
10 mice, involves superovulation of a donor female, removal of the eggs, injection of the foreign genes into one pronucleus (one-celled embryo) and transfer of the injected eggs into the oviducts of a pseudopregnant host. See, e.g., Wagner, U.S. Patent No. 4,873,191, PCT Publication No. WO92/11757, and Fodor, et al., U.S. Patent No. 5,624,837, all of which are herein incorporated by reference.

Another method for producing transgenic animals involves the genetic  
15 manipulation of embryonic stem cell lines. These cell lines are transformed with exogenous genetic material of interest and subsequently used to provide chimeric animals, which have germ cells comprising the exogenous genetic material. The chimeric animal is then bred to provide the transgenic animal. See, e.g., Wheeler, U.S. Patent  
20 5,523,226, herein incorporated by reference.

The immune system suppressor polypeptide includes complement  
restriction factors and enzymes or proteins and any combination thereof, that are expressed by mesenchymal stem cells present in the tissues of the transgenic animal and functions to inhibit or prevent hyperacute rejection of the genetically altered  
25 mesenchymal stem cells when transplanted into a recipient animal, e.g., human. As described above, such a polypeptide includes, but is not limited to, complement restriction factors, and enzymes or proteins, such as glycosyltransferases and any combination thereof that when expressed by mesenchymal stem cells, present a complement regulating protein for the recipient or otherwise cause a reduction in the

expression of a xenogeneic antigen normally present on the surface of the donor mesenchymal stem cell.

As above, suitable complement restriction factors are either cell-surface proteins which include, but are not limited to, DAF, MCP, CD59, CR1, CR2, and HRF; or restriction factors present in the serum which include, but are not limited to, factor H, C4-bp, S-protein, SP-40, 40 and combinations thereof. Preferably, the complement restriction factor is a cell-surface protein and includes, but is not limited to, DAF, MCP, CD59, CR1, CR2, HRF and combinations thereof. More preferred cell-surface complement restriction factors include DAF, CD59, MCP and combinations thereof.

Suitable proteins or enzymes that when expressed by the xenogeneic mesenchymal stem cells of transgenic animals are capable of reducing the expression of xenogeneic antigens, and thus reduce or inhibit hyperacute rejection of the xenogeneic cell when transplanted in a recipient include, but are not limited to, glycosyl transferases such as human alpha-1,2 fucosyltransferase (H-transferase) and human lysosomal alpha-galactosidase as described above.

Methods of producing transgenic mice and pigs containing complement restriction factors such as DAF and CD59 have been described in Cary et al., "Tissue Expression of human decay accelerating factor, a regulator of complement activation expressed in mice: A potential approach to inhibition of hyperacute xenograft rejection," *Transplantn. Proc.* 25: 400-401, 1993; Diamond et al., "Cell and tissue specific expression of a human CD59 minogene in transgenic mice", *Transplantn. Proc.* 26: 1239, 1994; Rosengard et al., "Tissue Expression of human complement inhibitor, decay-accelerating factor, in transgenic pigs-a potential approach for preventing xenograft rejection", *Transplantation* 59: 1325-1333, 1995; Fodor et al., "Expression of a functional human complement inhibitor in transgenic swine as an approach to prevent xenogeneic hyperacute organ rejection", *Proc. Natl. Acad. Sci. U.S.A.* 91: 11153-11157, 1994; Langford et al., "Production of pigs transgenic for human decay accelerating factor", *Transplantn. Proc.* 26: 1400-1401, 1994, and Sims, et al. U.S. Patent No. 5,705,732; all of which are herein incorporated by reference.

Methods of producing transgenic animals which express a human galactosyltransferase have been described in Chen et al., "Transgenic expression of human alpha 1,2 fucosyltransferase (H-transferase) prolongs mouse heart survival in an *ex vivo* model of xenograft rejection", *Transplantation* 65: 832-837, 1998; and Koike et al., "Introduction of alpha(1,2)fucosyltransferase and its effect on alpha-Gal epitopes in transgenic pig", *Xenotransplantation* 3: 81-86, 1996, both of which are herein incorporated by reference.

As stated above, methods for isolating mesenchymal stem cells are well-known in the art and are useful herein to isolate mesenchymal stem cells either from naturally occurring animals prior to transfection to produce mesenchymal stem cells incorporating one or more immune system suppressor polypeptides, or from transgenic animals having immune system suppressor polypeptides incorporated into their genomes. Various isolation procedures well known in the art can be employed to obtain the desired stem cells depending on the tissue source. For example, the isolation of human mesenchymal stem cells from bone marrow is described in Caplan et al., U.S. 5,486,359, herein incorporated by reference. As specifically set forth in Example 1 of the present description, when mesenchymal stem cells are obtained from the bone marrow of pigs or mice, typically the marrow from tibias and femurs is flushed out with the culture medium, alpha-Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS). Subsequently, the marrow cells suspended in the culture medium are dispersed by passing the suspension of cells through a syringe fitted with an 18 gauge needle.

A key feature in isolating mesenchymal stem cells from a tissue, e.g., bone marrow, is the selection of a culture medium that not only allows for expansion (reproduction) of the cells without differentiation, but also permits the selective adherence of mesenchymal stem cells to the surface of the culture dish or flask. As a result, mesenchymal stem cells can be isolated from other cells present in the bone marrow which include hematopoietic stem cells, red and white blood cells.

Culture media useful in isolation and the adherence of mesenchymal stem cells are well known in the art. For example, one such medium is alpha-MEM supplemented with 10% FBS which is used in the initial isolation procedure.

5 Once the bone marrow cells are dispersed, these cells are plated onto culture flasks using the same medium utilized in the initial isolation step. The mesenchymal stem cells are allowed to adhere to the surface of the flask, while non-adhered cells are removed by replacing the medium. The adhered mesenchymal stem cells are grown to confluence, which typically requires 7-10 days.

10 Isolated mesenchymal stem cells may be frozen in alpha-MEM supplemented with 10% FBS and 10% dimethyl sulfoxide (DMSO) at -80°C, and thawed for subsequent use, or used fresh.

The cells are detached from the culture plates by treatment with trypsin with EDTA (ethylene diaminetetra-acetic acid), and washed with medium.

15 Characterization of the cells as mesenchymal stem cells can be determined by three different methods, visualization of such cells using phase contrast microscopy, the alkaline phosphatase assay and expression assays for collagen I and II.

20 With phase contrast microscopy, mesenchymal stem cells when cultured at low density exhibit a flat appearance instead of a spindle-shaped fibroblast-like appearance. Thus, these cells can be readily distinguished from fibroblasts and other cells present in bone marrow and other sources of mesenchymal stem cells (see Example 2).

In the alkaline phosphatase assay, isolated cells are allowed to differentiate into osteoblasts. The osteoblasts express alkaline phosphatase, which can be detected at a wavelength of 405 nm using a plate reader, which in turn, indicates that the isolated cells are mesenchymal stem cells (see Example 2).

25 Another approach in characterizing the isolated cells as mesenchymal stem cells is to allow the isolated cells to differentiate into chondrocytes which express collagen I and II (see Example 2). These proteins can be detected using standard techniques known in the art such as immunofluorescence, and western blot as described in *Principles and Practice of Immunoassay*, Christopher P. Price and David J. Neoman

(eds), Stockton Press, New York, New York, 1991; and Ausabel et al. (ed), in *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, New York, 1987, both of which are incorporated herein by reference.

Expression of the immune system suppressor polypeptide can be detected  
5 by methods well known to one skilled in the art such as polymerase chain reaction, immunofluorescence, fluorescence activated cell sorting (FACS, also known as flow cytometry), and the human serum killing assay. Examples of the FACS method are set forth in Examples 3 and 4, and an example of the human serum killing assay is set forth in Example 3.

10 Once the isolated, genetically altered mesenchymal stem cells are grown to sufficient numbers, the cells can be induced to differentiate into specific types of desired tissue such as connective tissue, e.g., bone and cartilage, by a variety of different factors and/or specific conditions which are well known to those skilled in the art. Such factors and/or conditions are also referred to herein as "differentiation stimulating agents." For  
15 example, mesenchymal stem cells have been shown to differentiate into bone when incubated *in vivo* as a graft in porous ceramic cubes, which appears to allow accessibility of the cells to nutrients and growth factors supplied by the surrounding vasculature. See, e.g., Caplan et al., U.S. Patent No. 5,226,914, herein incorporated by reference. Various bioactive factors, e.g., platelet-derived growth factor-AA, insulin-like growth factor-I,  
20 have been shown to stimulate proliferation and/or differentiation of mesenchymal stem cells as described, e.g., in Young, "Stem Cells and Tissue Engineering," In: *Gene Therapy in Orthopaedic and Sports Medicine*, eds., Huard, J., and Fu, F., Springer-Verlag, New York, Inc., 1999, which is incorporated herein by reference.

25 Accordingly, the isolated, culturally expanded genetically altered stem cells can be used to treat a number of tissue disorders and tissue injuries such as those occurring in connective tissue disorders and tissue injuries in a recipient animal which includes humans, non-human primates and other animals. Preferably, the recipient of the donor cells is human.

Defects that can be treated using these cells include, but are not limited to, bone and joint fractures, bone defects resulting from trauma, tumor infection, tendon and ligament defects, muscle defects, and congenital defects.

5 Diseases which impair the ability of tissues to repair and reconstruct which can be treated using genetically altered mesenchymal stem cells described herein include, but are not limited to, muscular dystrophy, osteoarthritis, rheumatoid arthritis, and so forth.

10 Various therapeutic compositions including the isolated genetically altered mesenchymal stem cells and a pharmaceutically acceptable carrier are also contemplated for use in treating tissue in need of repair or reconstruction. The therapeutic compositions can include other cells in combination with the genetically altered mesenchymal stem cells. In addition, the therapeutic compositions can include one or more therapeutic agents, e.g., those which accelerate or beneficially modify the healing process. For example, the therapeutic agent can include, but is not limited to, 15 antimicrobial agents to aid in combating infections in a tissue repair or reconstruction site, e.g., broad spectrum antibiotics (gentamicin sulphate, erythromycin or derivatized glycopeptides); growth factors to aid in promoting repair and/or tissue growth, e.g., fibroblast growth factor, bone growth factor, epidermal growth factor, platelet derived growth factor, macrophage derived growth factor, alveolar derived growth factor, 20 monocyte derived growth factor, and so forth. Suitable pharmaceutically acceptable carriers include, but are not limited to, saline or a physiologically buffered solution, matrix proteins such as collagen and fibrin, and polymer and porous cube devices, which are described in more detail below.

25 Various methods are also contemplated for immobilizing, growing, delivering, and activating the genetically altered mesenchymal stem cells. These include but are not limited to subcutaneous implantation of the stem cells alone, or adhered to a porous ceramic cube or polymer device, or injection of the cells into muscle. See, e.g., Caplan et al, U.S. Patent 5,226,914, herein incorporated by reference.

For example, the isolated genetically altered mesenchymal stem cells can be used to repair skeletal defects caused by injury or produced when a large amount of cancerous bone tissue is removed, or in the case of non-union bone fractures, by implanting these cells in a calcium phosphate, hydroxyapatite, or other bioceramic device into the site of repair.

Various polymers useful in immobilizing, growing, delivering and activating the stem cells are contemplated and are well known in the art. These polymers include, but are not limited to, nonabsorbable polymers, e.g., polypropylene, polyethylene, nylon, polymethylmethacrylate, polyurethane, etc., and bioabsorbable polymers made of, e.g., glycolide, lactic acid, lactide, lactone, dioxanone,  $\epsilon$ -caprolactone, trimethylene carbonate, etc., and various combinations of these and related polymers as described, e.g., in Kennedy, U.S. Patent No. 5,102,983, which is incorporated herein by reference. Suitable bioabsorbable polymers also include, but are not limited to, lysine ethyl ester diisocyanate derivatives as described, e.g., in Bennett et al., U.S. patent 5,578,662, which is herein incorporated by reference.

Bioabsorbable polymers may be prepared in various forms, e.g., foams as described, e.g., in Kennedy, *supra*, and Bennett et al., *supra* (see Example 5). The foamed polymers may also be coated with matrix proteins, e.g., polylysine or fibronectin. In addition, bioadsorbable polymers can be combined with non-absorbable polymers or components. For example, fibers coated with bioabsorbable polymers can be knitted or woven with other fibers, either absorbable or nonabsorbable to form meshes, which may be coated with matrix proteins such as, e.g., polylysine or fibronectin (see Example 6).

The mesenchymal stem cells are also useful in repairing damaged articular cartilage caused by trauma or diseases such as osteoarthritis and rheumatoid arthritis, by implanting the cells directly or in combination with a carrier, e.g., collagen or fibrin, into the defect. The following examples are included for purposes of illustrating certain embodiments and are not intended to limit the scope of this disclosure.



**EXAMPLE 1****Isolation, Purification, and Growth of Mesenchymal Stem Cells**

Porcine femurs and tibias were obtained from Alexion Pharmaceuticals, Inc. (New Haven, CT). The pigs were either normal (non-transgenic) or transgenic expressing CD59.

Tibias and femurs were dissected from 8-10 week old pigs. The ends of the bones were cut, and the marrow was flushed out with 2 ml of ice-cold alpha-MEM medium (alpha medium-MEM: Minimum Essential Medium; Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Fetal Bovine Serum; Sigma Chemical Co., St. Louis, MO) by using a syringe with an 18 gauge needle (Becton Dickinson, Plymouth, England). The pooled marrow cells were dispersed by agitation in the syringe.

Nucleated bone marrow cells ( $5 \times 10^6$  to  $5 \times 10^7$ ) were resuspended in 10 ml of alpha-MEM supplemented with 10% FBS and plated onto 25 or 75-cm<sup>2</sup> culture flasks (Falcon, Becton Dickinson, Plymouth, England). After 4 hours to overnight, nonadherent cells were removed by replacing the medium. The adherent cells were expanded from  $5 \times 10^4$  to  $1 \times 10^7$  by culture for 7-10 days with replacement of the medium on day 7. Subsequently, the cells were resuspended in 10% DMSO (dimethyl sulfoxide; Sigma: St. Louis, MO) and 90% alpha-MEM containing 10% FBS, added to 2 ml cryogenic vials (Corning, Corning, NY) and frozen at -80°C until use.

A vial was removed from the freezer thawed at 37°C, and added to a 25 cm<sup>2</sup> culture flask (Falcon, Becton Dickinson, Plymouth, England). Cells were further expanded for use as described above.

## **EXAMPLE 2**

### **Characterization of the Isolated Cells as Mesenchymal Stem Cells**

#### **I. Phase Contrast Microscopy**

One approach in determining whether the cells isolated from porcine bone marrow were mesenchymal cells was by their morphology. As shown in Figure 1, almost all of the isolated cells exhibited a flat appearance rather than a spindle-shaped fibroblast-like morphology when cultured at low cell density, indicating that the cells were mesenchymal stem cells.

#### **II. Alkaline Phosphatase Assay**

Undifferentiated mesenchymal stem cells do not express alkaline phosphatase, but when differentiated into osteoblasts they express alkaline phosphatase. In order to determine whether the isolated cells from pigs' femurs were mesenchymal stem cells, the isolated cells were allowed to differentiate into osteoblasts, followed by determination of alkaline phosphatase.

Cells obtained from pigs' femurs were split into three different culture dishes having a different cell concentration in each culture dish: low cell concentration (less than  $1 \times 10^6$  cells, no cell-cell contact), medium cell concentration ( $2 \times 10^6$  cells, some cells were in contact), and high cell concentration ( $5 \times 10^6$  cells, all cells were in cell-cell contact). Primary bone culture cells which express alkaline phosphatase were used as a positive control. NIH 3T3 fibroblasts (ATCC, Rockville, MD) and uninduced MC-3T3 osteoblasts that did not express alkaline phosphatase were used as negative control cells.

Cells from the above culture dishes were removed by trypsin with EDTA, neutralized with a serum-containing medium (the same medium used for growing the cells). The cells were then washed three times with PBS (Phosphate Buffer Saline; Life Technologies; Gaithersburg, MD) and counted. Various cell concentrations were resuspended in 9:1 PNP:buffer A (Buffer A: 0.1M Tris-HCL, 0.2 M  $MgCl_2$  -Sigma Chemical Co., St. Louis, MO., 0.2% Nondiet P-40- Fluka Chemical Corp., Ronkonkoma,

NY, pH = 9.0; pNP: p-nitrophenyl phosphate; Sigma, St. Louis, MO.) in eppendorf tubes for 1 hour at 37°C. The materials were then transferred to a 96-well culture dish (Corning; Corning, NY), which was read at wavelength 405 nm using a plate reader (Model 3550-UV, Bio-Rad, Hercules, CA).

5                   As shown in Figure 2, both NIH 3T3 fibroblasts and uninduced MC-3T3 osteoblasts did not express any alkaline phosphatase activity. Primary cultures of osteoblasts expressed an increasing alkaline phosphatase activity with an increasing concentration of cells. Expression of alkaline phosphatase activity was detected in the high cell density culture dish, but this activity was lower than the activity detected in the  
10                   medium density culture dish. Low activity was detected in the low cell density culture dish and was similar to that observed in the NIH 3T3 medium. The above data demonstrated that the isolated cells from pigs were mesenchymal stem cells since they differentiated into osteoblasts under high cell-density.

### 15                   III. Assay for Expression of Collagen I and II:

Mesenchymal stem cells differentiate into chondrocytes expressing collagen I and II. Accordingly, another approach to determine whether isolated cells from pigs' femurs were mesenchymal stem cells was to allow the cells to differentiate into chondrocytes in culture and examine their expression of collagen I and II. Expression of  
20                   collagen I and II was examined by two different methods described below.

#### A. Indirect Immunofluorescence Assay:

Mesenchymal stem cells from the femurs of normal or transgenic pigs expressing CD59 were maintained at three different cell concentrations: low cell,  
25                   concentration (about  $1 \times 10^6$  cells, no cell-cell contact), medium cell concentration (about  $2 \times 10^6$  cells, some cells were in contact), and high cell concentration ( $5 \times 10^6$  cells, all cells were in cell-cell contact). Porcine chondrocytes (obtained from Alexion), which were known to express collagen I and II were used as positive control cells. NIH 3T3 cells were used as negative control cells.

The following primary antibodies were used at the following concentrations: polyclonal antibody to Collagen I - 1: 200 (Rockland, Gilbertsville, PA), polyclonal antibody to Collagen II - 1: 200 (Rockland, Gilbertsville, PA), and secondary antibody fluorescent goat anti- rabbit - 1: 300 (BioSource, Camarillo, CA).

5                   Glass coverslips 12 mm (Fisher, Pittsburgh, PA) were coated with 20  $\mu$ g/ml polylysine (Sigma: St. Louis, MO) for 4 hours in PBS at 37°C. Coverslips were washed with PBS three times. Subsequently, the cells were added to the coverslips in their serum-containing medium and were allowed to adhere to the coverslips for 1 week at 37°C in a 5% CO<sub>2</sub> incubator. After 1 week, the coverslips were washed with PBS  
10                   three times to remove non-adherent cells. A standard, immunofluorescence assay was then performed.

The first antibody was added at the concentration stated above in 10% goat serum (ICN: Costa Mesa, CA) in PBS for 1-2 hours at 37°C. The coverslips were then washed three times with PBS to remove nonadhered antibody.

15                   Secondary antibody was added at the concentration stated above in 10% goat serum in PBS for 1 hour at 37°C. The coverslips were washed three times with PBS to remove nonadhered antibody, and placed on slides with the cells facing down immersed in mounting medium (ICN: Costa Mesa, CA). The coverslips were examined under a fluorescent microscope (Zeiss: Thornwood, NY).

20                   Mesenchymal stem cells which differentiated into chondrocytes expressed collagen I and II similar to porcine chondrocytes used as a positive control.

#### **B. Western Blot Assay:**

25                   Mesenchymal stem cells were split into three different culture dishes: low cell concentration (1 x 10<sup>6</sup> cells, no cell-cell contact), medium cell concentration (2 x 10<sup>6</sup> cells, some cells were in contact), and high cell concentration (5 x 10<sup>6</sup> cells, all cells were in cell-cell contact). NIH 3T3 cells were used as negative control cells. Medium was removed from the different dishes and stored in the freezer for immunoblotting studies.

Adherent cells were washed twice with cold PBS. 0.5 ml of NP-40 0.5% in PBS with protease inhibitors was added to each plate and left on ice for 30 minutes. Protease inhibitors used (fc = final concentration) were: PMSF fc 2 mM (300  $\mu$ l of stock of 100 mM), trypsin inhibitor fc 60  $\mu$ g/ml (1 ml of stock 1 mg/Ml), leupeptin fc 30  
5  $\mu$ g/ml, pepstatin A fc 60  $\mu$ g/ml and aprotinin A fc 24  $\mu$ g/ml. All protease inhibitors were from Sigma Chemical Co. (St. Louis, MO).

The cells' extracts were transferred to eppendorf tubes, centrifuged at high table speed for 15 minutes and the supernatants were collected. Protein assays (Bio-Rad, Hercules, CA) were performed on the collected medium from various cultures and from  
10 cell extracts. Equal amounts of proteins from different mediums or cell extracts were run on 15% SDS-PAGE gel.

The following proteins were added to the SDS-PAGE gel as positive controls: 0.5-1  $\mu$ g collagen I or collagen II, 5-10  $\mu$ l of control medium for chondrocytes (not conditioned meaning no cells were grown on this medium) and 5-10  $\mu$ l conditioned  
15 medium for chondrocyte (chondrocytes were grown in this medium).

Subsequently, the proteins were transferred to nitrocellulose paper (Bio-Rad, Hercules, CA) using a transfer box (Bio-Rad, Hercules, CA). Anti-Collagen I or II at a concentration of 1/5000 were then added to strips of the nitrocellulose paper in 5% milk in PBS and 1% Tween 20. The strips and the solution were left on a shaker for  
20 1-2 hours. The strips were washed with PBS 4 x 15 minutes. The second antibody, HRP (horse radish peroxidase) Goat-anti-rabbit at a concentration of 1/10,000 was added to the strips similar to the first antibody. The strips were washed again with PBS three times, dried and enhanced using Enhanced Chemiluminescence (Fisher, Pittsburgh, PA). The gel was then exposed to X-film (Kodak, Rochester, NY) and processed in a film processor  
25 (Picker, Cherry Hill, NJ).

Mesenchymal stem cells at medium cell density differentiated into chondrocytes and expressed collagen I and II, which was detected by the appearance of a band on the nitrocellulose paper at the same molecular weight as the collagen I and collagen II control proteins.

### **EXAMPLE 3**

#### **Detection of CD59 in Mesenchymal Stem Cells Isolated from Transgenic Pig**

##### **I. FACS Analysis Of Mesenchymal Stem Cells Isolated from Transgenic Pig Expressing CD59**

5                   Mesenchymal stem cells were isolated from transgenic pigs expressing CD59, purified, culturally expanded, frozen, and characterized as set forth above in Examples 1 and 2.

                  A FACS Caliber Cell Sorter (Becton Dickinson: Plymouth, England) was used to sort out mesenchymal stem cells expressing CD59 from normal pigs. The  
10               following cells were utilized: mesenchymal stem cells from non-transgenic pigs and transgenic pigs expressing CD59 (pigs nos. 2, 3 and 4). The antibody utilized was ALP3 (rabbit polyclonal): concentration 1:100.

                  The cells (500,000/tube) were incubated for 1 hour in DMEM with 2% serum, and then washed two times with a serum-free medium (DMEM). Subsequently,  
15               the cells were incubated with secondary antibody (anti-mouse, or anti-rabbit): concentration 1:300 for 1 hour, and washed two times with a serum-free medium (DMEM). The cells were then analyzed using a FACS Caliber Cell Sorter (Becton Dickinson, Plymouth, England).

                  Figure 3 shows FACS profiles of porcine mesenchymal stem cells isolated  
20               from transgenic pigs expressing CD59 and non-transgenic pigs using ALP3 antibody. As shown in Figure 3, an increase in the mean fluorescence intensity of mesenchymal stem cells expressing CD59 isolated from transgenic pigs was obtained when compared to mesenchymal stem cell isolated from non-transgenic pigs. These results indicate that  
                  CD59 is expressed on the surface of the CD59 cell lines (from transgenic pigs nos. 2, 3  
25               and 4) and is recognized by the ALP3 antibody. All three CD59 cell lines showed similar levels of expression of CD59.

## II. Complement-Mediated Dye Release Assay: Analysis of Dye Release by FACS

The detection of CD59 on the surface of transgenic porcine mesenchymal stem cells and the ability of CD59 expressed on these cells to regulate complement mediated cytotoxicity was determined using a complement-mediated dye release assay. The assay  
5 relied on hydrolysis by an intracellular esterase of calcein-AM (Molecular Probes, Inc.), a virtually non-fluorescent and membrane-permeable esterase substrate to the fluorescent calcein molecule. Calcein is a poly-anionic molecule that is retained in the living cell and produces an intense uniform green fluorescence.

Transgenic porcine mesenchymal stem cells expressing CD59 were isolated as  
10 described above. The transgenic cells and control litter mate cell were loaded with dye by incubation with calcein. Subsequently, the cells were incubated at room temperature in 1 X HBSS, 1% BSA, and complement activating anti-porcine polyclonal antisera (Inter-Cell Technologies). The cells were then incubated at 37°C for 30 minutes with varying amount of human whole serum. The cells were then assayed on a FACS Caliber Cell  
15 Sorter (Becton Dickinson, Plymouth, England). The percent dye release was measured by determining the loss of fluorescence (indicates cell damage) which was visualized in the FL1 channel.

The results as shown in Figures 4A (low cell density) and 4B (high cell density) demonstrate that CD59 was expressed on the surface of porcine transgenic  
20 mesenchymal stem cells, and that the expression of CD59 on the surface of these cells protected these cells from lysis by human serum. In contrast, control litter mate cells not expressing CD59 were sensitive to human serum lysis.

## **EXAMPLE 4**

### **Mesenchymal Stem Cells From Non-Transgenic Pigs Transfected With Vectors Carrying Human H-Transferase Gene**

#### **I. Determination of Neomycin Resistance for Mesenchymal Stem Cells**

5           The killing curve of neomycin drug G418 for mesenchymal stem cells prepared from pig's femurs was determined, and the ideal concentration of G418 was used in isolating the transfected mesenchymal stem cells.

          The following types of cells were trypsinized, neutralized and suspended in their complete medium and added to a 24-well culture dish (Corning: Corning, NY):  
10       NIH 3T3 cells, mesenchymal stem cells, and MC 3T3 cells (P12). The cells were allowed to adhere to plates overnight.

          Increasing concentrations of G418 (Geneticin; Life Technologies (Gibco/BRL): Gaithersburg, MD) were added to the cells for 6 weeks at 37°C. The concentrations of G418 used were: 50, 100, 300, 400, 500, 600 and 1200  $\mu\text{g/ml}$  and one  
15       well without the drug. Five weeks later, the wells were washed, fixed and stained with Coomassie blue.

          No cells were seen at concentrations of 400  $\mu\text{g/ml}$  and higher suggesting that this concentration of G418 killed cells that did not have neomycin resistance. Using this concentration, cells transfected with human H-transferase as described below were  
20       selected i.e., cells that were transfected survived and those that were not transfected died.

#### **II. Transfection of Porcine Mesenchymal Stem Cells from Non-Transgenic Pigs with Human H-Transferase**

          Mesenchymal stem cells isolated from normal pigs were seeded on 2 x 60  
25       mm culture dishes (Corning: Corning, NY) at 50-60% confluence for overnight. Two culture dishes were transfected either with human H-transferase or with the vector alone. Transfected cells were obtained from Alexion Pharmaceuticals, Inc., New Haven, CT. Two culture plates with cells were not transfected.



The viral supernatant (obtained from Alexion) was diluted at 1: 5, and 2.5 ml of the diluted supernatant was added to the plates and 1  $\mu$ l/ml of Polybrene (sterile stock 8 mg/ml = 10000x) was added to the medium. The culture dishes were incubated overnight at 37°C.

5                   The dishes were then washed with a serum-free DMEM three times. The cells were trypsinized and transferred to 100 mm culture dishes (Corning: Corning, NY). The cells were allowed to recover for 2-3 days followed by the addition of G418 (400  $\mu$ g/ml). The medium was changed every week.

10                   After 2 weeks, all cells in the non-transferred dishes died suggesting that the concentration of G418 used did not allow for any cells to grow.

                  There were many cells left in both vector alone or human H-transferase transfected dishes after 3 weeks of neomycin treatment.

### III. Detection of Expression of Human H-Transferase in Transfected Cells

15                   The determination that transfected cells express human H-transferase was carried out by immunofluorescence and FACS.

#### A. Immunofluorescence

20                   The following cells were utilized in the immunofluorescence assay: mesenchymal stem cells from normal pig that were not transfected, mesenchymal stem cells from normal pig transfected with human H-transferase, and control cells, HUVEC endothelial cells.

25                   Glass coverslips (Fisher, Pittsburgh, PA) were coated with 20 $\mu$ g/M1 Poly-D-Lysine (Sigma Chemical Co., St. Louis, MO) in a serum-free medium (DMEM) for 4 hours at 37°C, then with 10  $\mu$ g/ml fibronectin (Sigma Chemical Co., St. Louis, MO), and then left overnight in a serum free medium (DMEM) at 37°C. Coverslips were then washed with DMEM three times. Cells were added to the coverslips in their serum-containing medium at a cell concentration of 1000 cells/coverslip. There were two

coverslips each for: human H-transferase marker, UEA-1(lectin) (EY Laboratories, Inc., San Mateo, CA), and for secondary antibody alone.

A standard immunofluorescence assay was performed as set forth in Example 2. The antibodies used were: first Ab conc.: human H-transferase marker  
5 UEA-1: concentration 1:100.

HUVEC cells (positive control) were labeled with the above antibodies. The mesenchymal stem cells transfected with human H-transferase were labeled with human H-transferase marker, UEA-1 (Figure 5A), indicating expression of human H-transferase on the surface of the mesenchymal stem cells. The mesenchymal stem cells  
10 transfected with vector only (negative control) were not labeled with UEA-1 (Figure 5B).

## **B. FACS Analysis and Sorting**

### **1. Analysis**

A FACS Caliber Cell Sorter (Becton Dickinson, Plymouth, England) was  
15 used to sort out mesenchymal stem cells from normal pigs that express any of the above markers. The following cells were utilized: mesenchymal stem cells from normal pig transfected with human H-transferase and mesenchymal stem cells from normal pig transfected with vector alone.

Cells were removed by trypsinization and neutralized with a serum-containing medium. The experiment was performed in PBS plus 10% normal goat serum  
20 for 2 hours at room temperature. The cells were washed three times with PBS at low speed, and processed for immunofluorescence assay as described above using the human H-transferase marker, UEA-1 (lectin): 1:100. Subsequently, the cells were transferred to tubes with 4  $\mu$ g/ml of Propidium Iodide and left on ice until reading. The cells were  
25 then analyzed using a FACS Calibur Cell Sorter (Becton Dickinson: Plymouth, England).

Figure 6 shows FACS profiles of porcine mesenchymal stem cells transfected with vector carrying the H-transferase gene relative to cells transfected with vector alone. As shown in Figure 6 an increase in the mean fluorescence intensity of

mesenchymal stem cells transfected with human H-transferase was obtained when compared to mesenchymal stem cells transfected with vector alone. The mean fluorescent intensity for each porcine cell type is shown in Table 1.

5

**TABLE 1**

Cell Type	Mean Fluorescent Intensity
Vector alone - Porcine	4.01
H-transferase Transfected-Porcine	53.72

10

These results, in turn, indicate that H-transferase is expressed on the surface of transfected porcine mesenchymal stem cells and is recognized by human H-transferase monoclonal antibody.

## 2. Sorting

15

The following cells were utilized: mesenchymal stem cells from normal pig transfected with human H-transferase and mesenchymal stem cells from normal pig transfected with vector alone.

20

Cells were washed with a serum-free medium (DMEM) three times, removed by trypsinization and neutralized with a serum-containing medium, washed again with a serum-free medium DMEM once. The marker used was human H-transferase marker, UEA-1 (lectin): 1:25. The experiment was performed in a 2 % serum-containing medium for 2.0 hours at room temperature. The cells were then washed with a serum free medium, DMEM, three times at room temperature (low speed). No Propidium Iodide was added to the cells to be sorted.

25

The cells were sorted using a FACS Calibur Cell Sorter (Becton Dickinson: Plymouth, England). Three gates were set for sorting: M1 yielded 100,000 cells-contained all expressors, M2 yielded 50,000 cells-contained medium expressors, M3 yielded 10,000 cells-contained high expressors. After sorting, cells were centrifuged and resuspended in DMEM. Sorted cells were transferred to a 10 cm culture dish and a

serum-containing medium was added. The medium was changed every 2-3 days until confluence was reached.

The cells collected in a serum-coated tube looked healthy, whereas those collected in the uncoated tubes showed no viability. Sorted positive mesenchymal stem cells transfected with H-transferase adhered and proliferated in their serum-containing medium on a culture dish.

As shown in the FACS profile illustrated in Figure 7, there was an increase in fluorescence intensity of human H-transferase transfected cells after two rounds of sorting compared to human H-transferase transfected cells before two rounds of sorting. The mean fluorescence intensity value for human H-transferase transfected cells before and after two rounds of sorting as shown in Table 2.

**TABLE 2**

Cell Type	Mean Fluorescent Intensity
H-transferase Transfected (pool) - Porcine	53.72
Sorted H-transferase Expressors - Porcine	84.72

These results show that cells obtained after two rounds of sorting expressed higher levels of human H-transferase compared with cells obtained before two rounds of sorting.

**C. Expression of Human H-transferase and Gal alpha(1,3) Gal epitope on Normal and Transfected Porcine Mesenchymal Stem Cells, and Normal Monkey and Rabbit Mesenchymal Stem Cells**

This experiment was performed to measure expression of the Gal alpha(1,3) Gal epitope in porcine mesenchymal stem cells transfected with human H-transferase.

The following cells were utilized in this experiment: porcine mesenchymal stem cells (second sort) transfected with human H-transferase, porcine mesenchymal stem cells transfected with vector alone, normal (non-transfected) rabbit and monkey

mesenchymal stem cells. Cells were washed with a serum-free medium DMEM three times, removed by trypsinization and neutralized with a serum-containing medium, and then washed again with the serum-free medium DMEM.

A standard immunofluorescence assay was performed as set forth in Example 2. The following primary antibodies were used: HT Marker UEA-1 (lectin): 1:50 or 1B4: 1:50 (FITC-conjugated lectin), which is specific for the Gal alpha(1,3) Gal cell-surface epitope. The experiment was performed in a 2% serum-containing medium for 2 hours at room temperature. Cells were washed three times with a serum-free medium, DMEM, at room temperature (low speed), centrifuged, and resuspended in a serum-free medium. Propidium Iodide was added, and FACS analysis was performed using a FACS Calibur Cell Sorter (Becton Dickinson: Plymouth, England)..

As shown in the FACS profile illustrated in Figure 8, there was a definite shift (an increase) in fluorescence intensity of human H-transferase transfected porcine mesenchymal stem cells relative to porcine mesenchymal stem cells transfected with vector alone. As shown in Table 3, the mean fluorescence intensity value of human H-transferase transfected porcine mesenchymal stem cells (62.65) approached the mean fluorescence intensity value obtained with normal (non-transfected) monkey mesenchymal stem cells (60.36).

**TABLE 3**

Cell Type	Mean	Geometric Mean
Vector alone - Porcine	3.96	3.60
H-transferase Transfected - Porcine	62.65	16.75
Rabbit	9.12	6.11
Monkey	60.36	23.09

These results indicate that porcine mesenchymal stem cells transfected with human H-transferase expressed human H-transferase on their cell surface at a level similar to that observed with normal monkey mesenchymal stem cells.

Figure 9 is a FACS profile, which shows a definite shift (a decrease) in mean fluorescence intensity of GaP alpha (1, 3) Gap in human H-transferase transfected porcine mesenchymal stem cells relative to porcine mesenchymal stem cells transfected with vector alone. The mean fluorescence intensity values for each cell type are shown in Table 4.

**TABLE 4**

Cell Type	Mean	Geometric Mean
Vector alone - Porcine	163	120
H-transferase Transfected - Porcine	66.1	28.6
Rabbit	94	59
Monkey	17.4	9.4

These results indicate that porcine mesenchymal stem cells transfected with human H-transferase expressed reduced levels of the Gal alpha(1,3) Gal epitope compared with expression of this epitope in non-transfected porcine mesenchymal stem cells. The level of expression of the Gal alpha(1,3)Gal epitope on the surface of transfected porcine mesenchymal stem cells approached that observed on the surface of normal monkey mesenchymal stem cells.

**EXAMPLE 5****Adherence of Mesenchymal Stem Cells on Degradable  
Lysine Ethyl Ester Diisocyanate Foams**

Three types of LDI foams as described in U.S. Patent 5,578,662, with different pore size (small, medium and large) were coated with either 10  $\mu$ g/ml of h-fibronectin (Sigma Chemical Co., St. Louis, MO) or 10  $\mu$ g/ml of polylysine (Sigma Chemical Co., St. Louis, MO). The foams were then incubated with a serum-containing medium for 30 minutes. Porcine mesenchymal stem cells ( $100-250 \times 10^3$  cells)

transfected with human H-transferase and non-transfected porcine mesenchymal stem cells ( $100-250 \times 10^3$  cells) were trypsinized, neutralized with a serum-containing medium and resuspended in serum-containing medium. The foams and transfected cells were added to scintillation vials and shaken slowly at  $37^\circ\text{C}$  for 1 week. Subsequently, the foams were transferred to 6 well culture dishes, and trypsinized to release the adherent cells. The following day, foams were transferred to a new 6 well culture dishes and cells that adhered to the first set of culture dishes were fixed and stained with Coomassie blue.

Transfected and non-transfected mesenchymal stem cells released from polylysine-coated foams did not adhere to culture dishes while those released from fibronectin-coated foams did adhere to culture dishes. In examining the number of mesenchymal stem cells released from fibronectin-coated foams, it appeared that the foams with the largest pores exhibited the largest number of mesenchymal stem cells.

The adherence of mesenchymal stem cells to the above describe LDI degradable foams was determined upon coating the foams with the following matrix molecules: extracellular, matrix protein, fibronectin, and collagen II. Porcine mesenchymal stem cells ( $7.5 \times 10^5$ ) were grown for 3 months in glass vials to eliminate cell migration off the foams. The foam/cell mixtures were fixed in 10% formalin, embedded in paraffin without ethanol dehydration, sectioned ( $8 \mu\text{m}$  thick), deparaffinized, and stained with Hematoxin and Eosin. As shown in table 5, the cells adhered only on foams coated with fibronectin.

**TABLE 5**

Cells Seeded	Matrix Used	Ease of Sectioning	Number of Cells per Foam
--	--	--	--
MSC	--	--	--
MSC	ECM	--	--
MSC	Col II	--	--
MSC	FN	++	+

**EXAMPLE 6****Growth and Staining of Cells Grown on a Glycolide/Lactide Mesh**

Porcine mesenchymal stem cells or NIH 3T3 cells were seeded onto a  
5 mesh made of woven 18/82 glycolide-lactide copolymer as described in U.S. Patent  
5,393,594, which is incorporated herein by reference. The cells were allowed to grow for  
8 days in glass vials. Sections of these meshes were stained using different techniques in  
order to determine which method yielded the best cell visibility with low polymer  
staining. A simple Hematoxin and Eosin staining gave good results with low  
10 background. Figure 10 (photomicrograph) shows the growth of porcine mesenchymal  
stem cells on melted mesh for 2 hours (Figure 10A) and 3 days (Figure 10B). Figure  
11A and 11B (photomicrograph at 10x and 40x, respectively) shows the growth of  
porcine mesenchymal stem cells on mesh for 8 days. Figure 12A (photomicrograph at  
100x) shows mesh without mesenchymal stem cells. Figure 12B (photomicrograph at  
15 100x) shows the growth of mesenchymal stem cells on mesh for 3 days.

It will be understood that various modifications may be made to the  
embodiments disclosed herein. Therefore, the above description should not be construed  
as limiting, but merely as exemplifications of preferred embodiments. Those skilled in  
the art will envision other modifications within the scope and spirit of the claims  
20 appended hereto.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Tawil, Nabil  
Geis, David  
Gruskin, Elliott
- (ii) TITLE OF INVENTION: GENETICALLY ALTERED MESENCHYMAL STEM  
CELLS AND METHODS OF USE THEREOF
- 10 (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: Dilworth & Barrese  
(B) STREET: 333 Earle Ovington Boulevard  
(C) CITY: Uniondale  
(D) STATE: New York  
(E) COUNTRY: USA  
(F) ZIP: 11553
- (v) COMPUTER READABLE FORM:
- 20 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
- 25 (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Steen, Jeffrey S.
- (ix) TELECOMMUNICATION INFORMATION:
- 30 (A) TELEPHONE: 516-228-8484  
(B) TELEFAX: 516-228-8516

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1174 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	CAAGCAGCTC GGCCATGTGG CTCCGGAGCC ATCGTCAGCT CTGCCTGGCC TTCCTGCTAG	60
	TCTGTGTCCT CTCGTGAATC TTCTTCCTCC ATATCCATCA AGACAGCTTT CCACATGGCC	120
5	TAGGCCTGTC GATCCTGTGT CCAGACCGCC GCCTGGTGAC ACCCCCAGTG GCCATCTTCT	180
	GCCTGCCGGG TACTGCGATG GGCCCCAACG CCTCCTCTTC CTGTCCCCAG CACCCTGCTT	240
15	CCCTCTCCGG CACCTGGACT GTCTACCCA ATGGCCGGTT TGGTAATCAG ATGGGACAGT	300
	ATGCCACGCT GCTGGCTCTG GCCCAGCTCA ACGGCCGCGG GGCCTTTATC CTGCCTGCCA	360
	TGCATGCCGC CTTGGCCCCG GTATTCCGCA TCACCCTGCC CGTGCTGGCC CCAGAAGTGG	420
20	ACAGCCGCAC GCCGTGGCGG GAGCTGCAGC TTCACGACTG GATGTCGGAG GAGTACGCGG	480
	ACTTGAGAGA TCCTTTCCTG AAGCTCTCTG GCTTCCCCTG CTCTTGACT TTCTCCACC	540
25	ATCTCCGGA ACAGATCCGC AGAGAGTTCA CCCTGCACGA CCACCTTCGG GAAGAGGCGC	600
	AGAGTGTGCT GGGTCAGCTC CGCCTGGGCC GCACAGGGGA CCGCCCGCGC ACCTTTGTCTG	660
30	GCGTCCACGT GCGCCGTGGG GACTATCTGC AGGTTATGCC TCAGCGCTGG AAGGGTGTGG	720

TGGGCGACAG CGCCTACCTC CGGCAGGCCA TGGACTGGTT CCGGGCACGG CACGAAGCCC 780  
 CCGTTTTTCGT GGTCAACGAG AACGGCATGG AGTGGTGTA AGAAAACATC GACACCTCCC 840  
 5 AGGGCGATGT GACGTTTGCT GGCGATGGAC AGGAGGCTAC ACCGTGGAAA GACTTTGCCC 900  
 TGCTCACACA GTGCAACCAC ACCATTATGA CCATTGGCAC CTTGGGCTTC TGGGCTGCCT 960  
 ACCTGGCTGG CGGAGACACT GTCTACCTGG CCAACTTCAC CCTGCCAGAC TCTGAGTTCC 1020  
 10 TGAAGATCTT TAAGCCGGAG GCGGCCTTCC TGCCCGAGTG GGTGGGCATT AATGCAGACT 1080  
 TGTCTCCACT CTGGACATTG GCTAAGCCTT GAGAGCCAGG GAGACTTTCT GAAGTAGCCT 1140  
 15 GATCTTTCTA GAGCCAGCAG TACGTGGCTT CAGA 1174

## (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
 20 (A) LENGTH: 365 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: unknown

25 (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Trp Leu Arg Ser His Arg Gln Leu Cys Leu Ala Phe Leu Leu Val  
 30 1 5 10 15  
 Cys Val Leu Ser Val Ile Phe Phe Leu His Ile His Gln Asp Ser Phe  
 20 25 30

	Pro His Gly Leu Gly Leu Ser Ile Leu Cys Pro Asp Arg Arg Leu Val
	35 40 45
5	Thr Pro Pro Val Ala Ile Phe Cys Leu Pro Gly Thr Ala Met Gly Pro
	50 55 60
	Asn Ala Ser Ser Ser Cys Pro Gln His Pro Ala Ser Leu Ser Gly Thr
	65 70 75 80
10	Trp Thr Val Tyr Pro Asn Gly Arg Phe Gly Asn Gln Met Gly Gln Tyr
	85 90 95
	Ala Thr Leu Leu Ala Leu Ala Gln Leu Asn Gly Arg Arg Ala Phe Ile
15	100 105 110
	Leu Pro Ala Met His Ala Ala Leu Ala Pro Val Phe Arg Ile Thr Leu
	115 120 125
20	Pro Val Leu Ala Pro Glu Val Asp Ser Arg Thr Pro Trp Arg Glu Leu
	130 135 140
	Gln Leu His Asp Trp Met Ser Glu Glu Tyr Ala Asp Leu Arg Asp Pro
	145 150 155 160
25	Phe Leu Lys Leu Ser Gly Phe Pro Cys Ser Trp Thr Phe Phe His His
	165 170 175
	Leu Arg Glu Gln Ile Arg Arg Glu Phe Thr Leu His Asp His Leu Arg
30	180 185 190
	Glu Glu Ala Gln Ser Val Leu Gly Gln Leu Arg Leu Gly Arg Thr Gly
	195 200 205

	Asp Arg Pro Arg Thr Phe Val Gly Val His Val Arg Arg Gly Asp Tyr	
	210	215 220
5	Leu Gln Val Met Pro Gln Arg Trp Lys Gly Val Val Gly Asp Ser Ala	
	225	230 235 240
	Tyr Leu Arg Gln Ala Met Asp Trp Phe Arg Ala Arg His Glu Ala Pro	
	245	250 255
10	Val Phe Val Val Thr Ser Asn Gly Met Glu Trp Cys Lys Glu Asn Ile	
	260	265 270
	Asp Thr Ser Gln Gly Asp Val Thr Phe Ala Gly Asp Gly Gln Glu Ala	
	275	280 285
15	Thr Pro Trp Lys Asp Phe Ala Leu Leu Thr Gln Cys Asn His Thr Ile	
	290	295 300
	Met Thr Ile Gly Thr Phe Gly Phe Trp Ala Ala Tyr Leu Ala Gly Gly	
20	305	310 315 320
	Asp Thr Val Tyr Leu Ala Asn Phe Thr Leu Pro Asp Ser Glu Phe Leu	
	325	330 335
25	Lys Ile Phe Lys Pro Glu Ala Ala Phe Leu Pro Glu Trp Val Gly Ile	
	340	345 350
	Asn Ala Asp Leu Ser Pro Leu Trp Thr Leu Ala Lys Pro	
	355	360 365
30		

**WHAT IS CLAIMED IS:**

1. An isolated mesenchymal stem cell comprising nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response to the mesenchymal stem cell in the recipient.  
5
2. An isolated mesenchymal stem cell according to claim 1 wherein the immune system suppressor polypeptide is a complement restriction factor selected from the group consisting of DAF, MCP, CD59, CR1, CR2, HRF, factor H, C1 inhibitor, C4- bp, S-protein, SP-40,40 and combinations thereof.  
10
3. An isolated mesenchymal stem cell according to claim 2 wherein the immune system suppressor polypeptide is a cell-surface complement restriction factor selected from the group consisting of DAF, CD59, MCP and combinations thereof.  
15
4. A composition comprising an isolated mesenchymal stem cell including nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response to the mesenchymal stem cell in the recipient, and a culture medium for growth, which allows the reproduction of the mesenchymal stem cell.  
20
5. A therapeutic composition for treating a tissue in need of repair or reconstruction comprising an isolated mesenchymal stem cell including nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response to the mesenchymal stem cell in the recipient, and a pharmaceutically acceptable carrier, the mesenchymal stem cell being present in an amount effective to aid in repair or reconstruction of the tissue by differentiating into a cell which is normally indigenous to the tissue.  
25

6. A therapeutic composition according to claim 5 wherein the cell which is normally indigenous to the tissue is selected from the group consisting of bone, cartilage, muscle, tendon and ligament.

5 7. A method of treating tissue in need of repair or reconstruction comprising administering to a subject having such tissue an effective repair or reconstructing amount of a therapeutic composition including an isolated mesenchymal stem cell including nucleic acid encoding an immune system suppressor polypeptide which is active in a recipient of the mesenchymal stem cell to inhibit an immune response  
10 to the mesenchymal stem cell in the recipient, and a pharmaceutical acceptable carrier, sufficient to treat the tissue.

8. A method of treating tissue in need of repair or reconstruction according to claim 7 wherein the isolated mesenchymal stem cell is mixed with a marrow sample from the subject, and the marrow sample containing the mesenchymal stem cells  
15 is reintroduced into the subject at a suitable locus.

9. A method of treating a tissue in need of repair or reconstruction according to claim 7 wherein the therapeutic composition is administered under  
20 conditions favoring differentiation of the mesenchymal stem cell into a cell selected from the group consisting of bone, cartilage, muscle, tendon and ligament.

10. A method of treating tissue in need of repair or reconstruction according to claim 7 wherein the therapeutic composition is administered to the subject  
25 by injection.

11. A method of treating tissue in need of repair or reconstruction according to claim 7 wherein the therapeutic composition is administered to the subject by implanting the therapeutic composition contained in a device into a defect site.

12. A method of treating tissue in need of repair or reconstruction according to claim 11 wherein the device is a bioabsorbable polymer foam.

5 13. An isolated mesenchymal stem cell according to claim 1 wherein the mesenchymal stem cell is obtained from a mammal.

14. An isolated mesenchymal stem cell according to claim 13 wherein the mammal is selected from the group consisting of pig, mouse, sheep, cow, goat and rabbit.

10

15. An isolated mesenchymal stem cell according to claim 2 wherein the complement restriction factor is CD59.

15 16. An isolated mesenchymal stem cell according to claim 2 wherein the complement restriction factor is a chimeric protein.

17. An isolated mesenchymal stem cell according to claim 1, wherein the immune system suppressor polypeptide is a glycosyltransferase.

20 18. An isolated mesenchymal stem cell according to claim 17 wherein the glycosyltransferase is selected from the group consisting of human H-transferase and human lysosomal alpha-galactosidase.

25 19. A method of producing a mesenchymal stem cell which does not trigger an immune response in a recipient of the mesenchymal stem cell comprising:  
isolating a mesenchymal stem cell; and  
introducing a vector including nucleic acid which encodes for an immune system suppressor polypeptide into the isolated mesenchymal stem cell, to provide the genetically altered mesenchymal stem cell.



20. A method of producing a mesenchymal stem cell which does not trigger an immune response in a recipient of the mesenchymal stem cell according to claim 19 wherein the nucleic acid is contained in a vector.

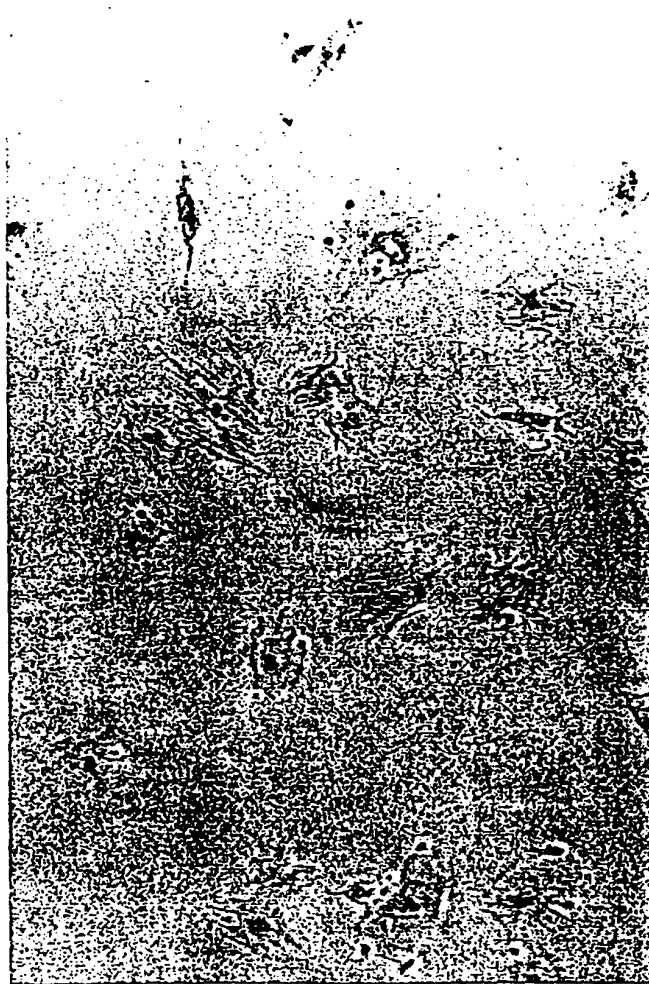
5                   21. A method of obtaining a mesenchymal stem cell which does not trigger an immune response in a recipient of the mesenchymal stem cell comprising providing a transgenic animal which has been engineered to produce cells having an immune system suppressor polypeptide, the immune system suppressor polypeptide adapted to reduce or inhibit an immune response to cells from the transgenic animal in  
10 the recipient, and isolating the mesenchymal stem cell from the transgenic animal.

                  22. A method of obtaining a mesenchymal stem cell which does not trigger an immune response in a recipient of the mesenchymal stem cell according to claim 21 wherein the mesenchymal stem cell is isolated from a source selected from the  
15 group consisting of bone marrow, blood, periosteum, dermis, and other tissues of mesodermal origin.

                  23. A method of obtaining a mesenchymal stem cell which does not trigger an immune response in a recipient of the mesenchymal stem cell according to  
20 claim 21 wherein the transgenic animal is selected from the group consisting of pig, mouse, sheep, cow, goat and rabbit.

                  24. A therapeutic composition according to claim 5 wherein the pharmaceutically acceptable carrier includes a bioabsorbable polymer foam.  
25

                  25. A therapeutic composition according to claim 24 wherein the bioabsorbable polymer foam is lysine ethyl ester diisocyanate.



**FIGURE 1**

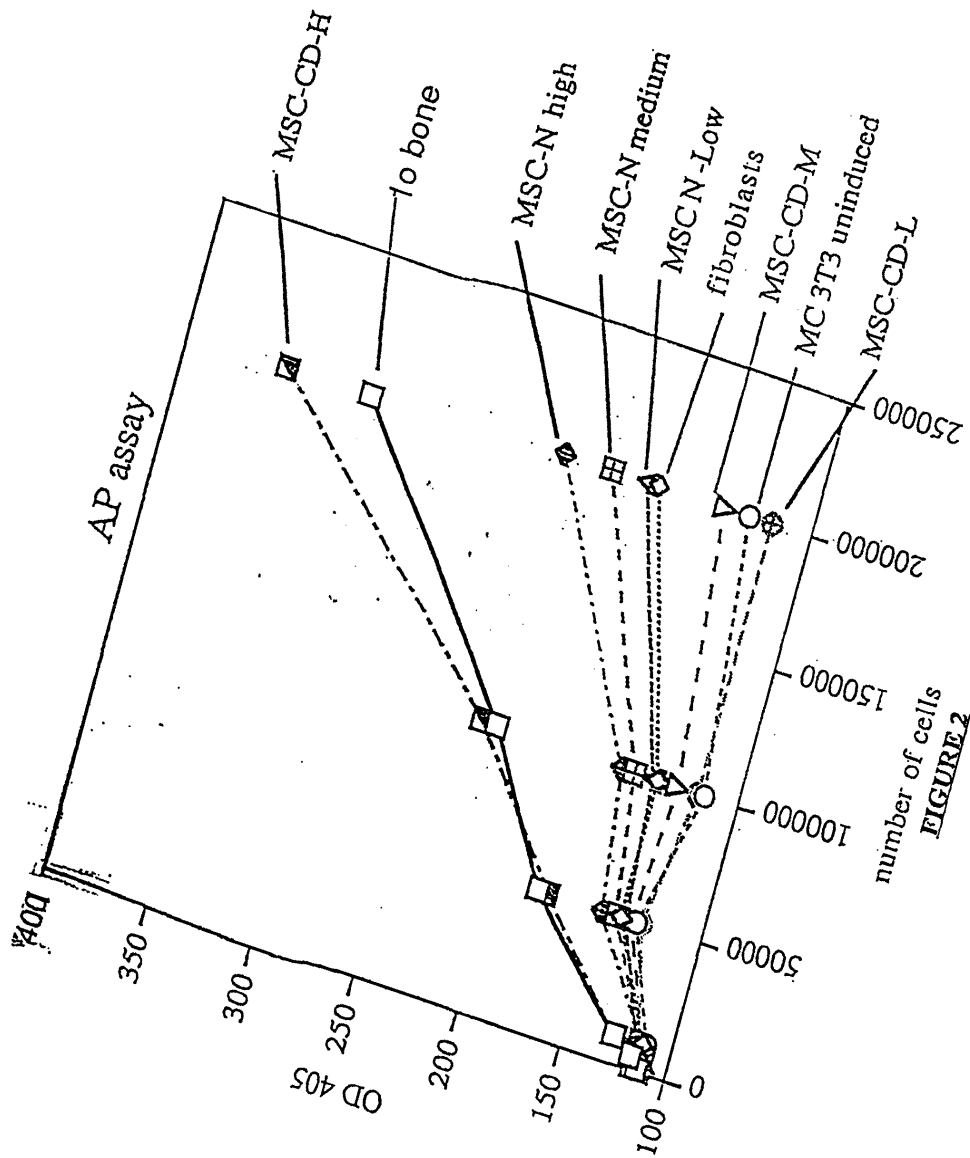
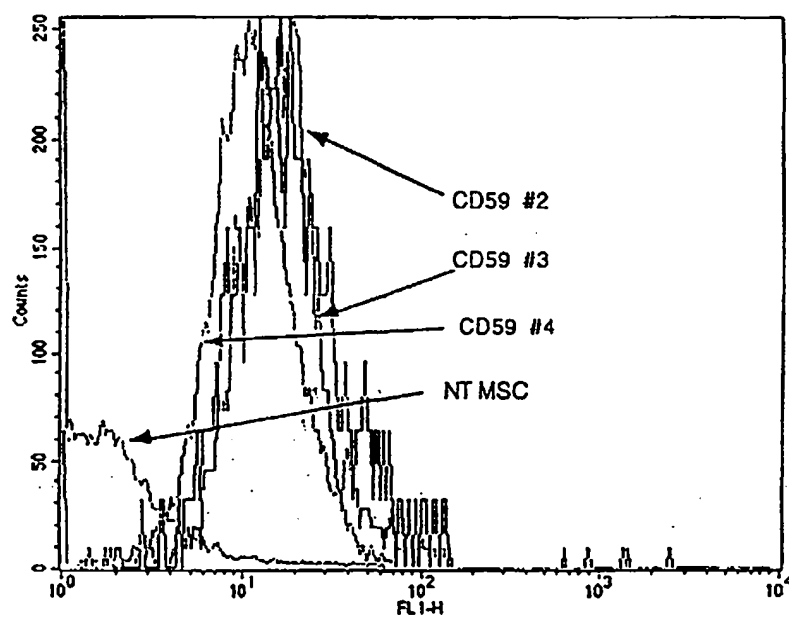
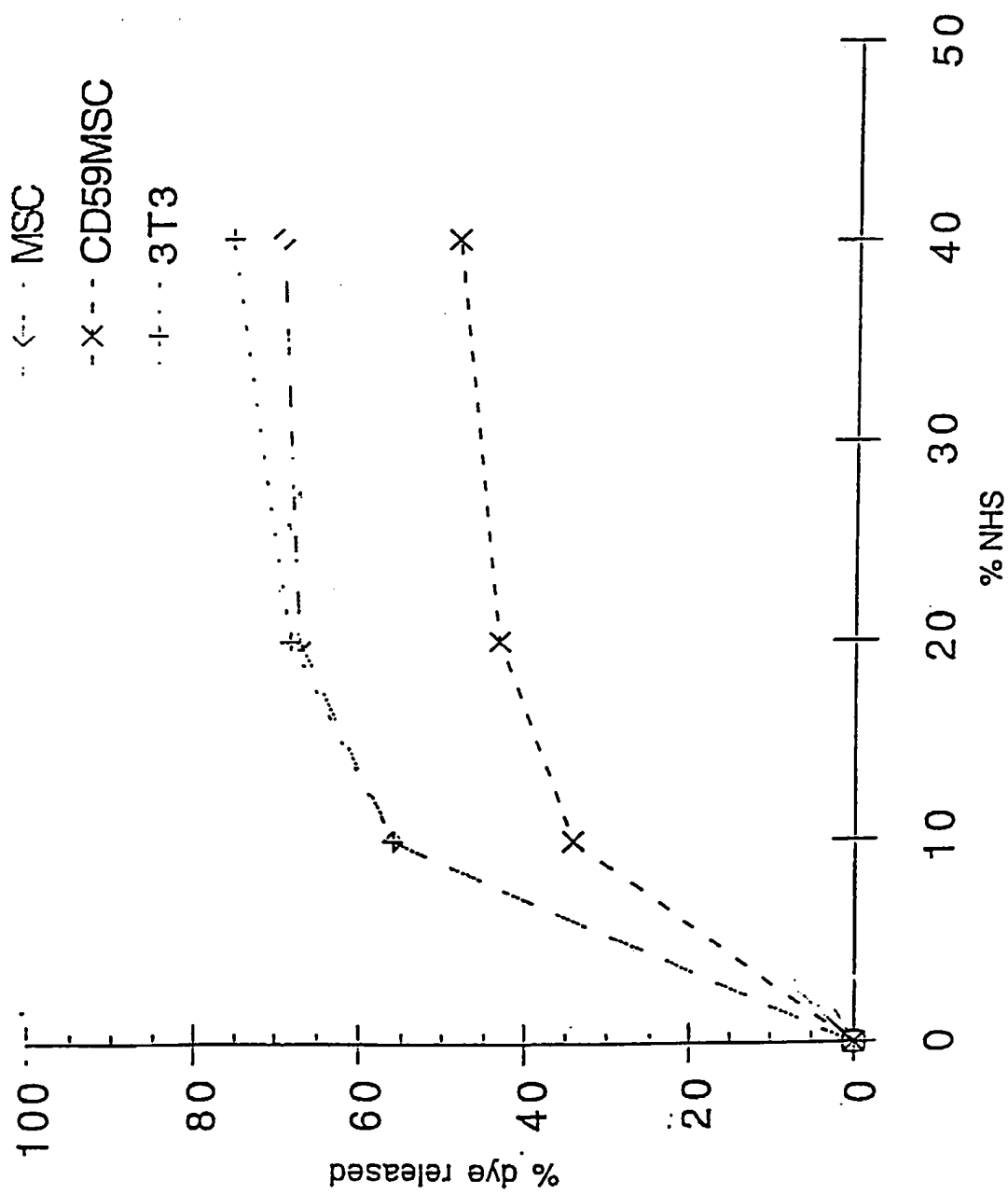


FIGURE 2

**FIGURE 3**

**FIGURE 4A**

5/13

**FIGURE 4B**

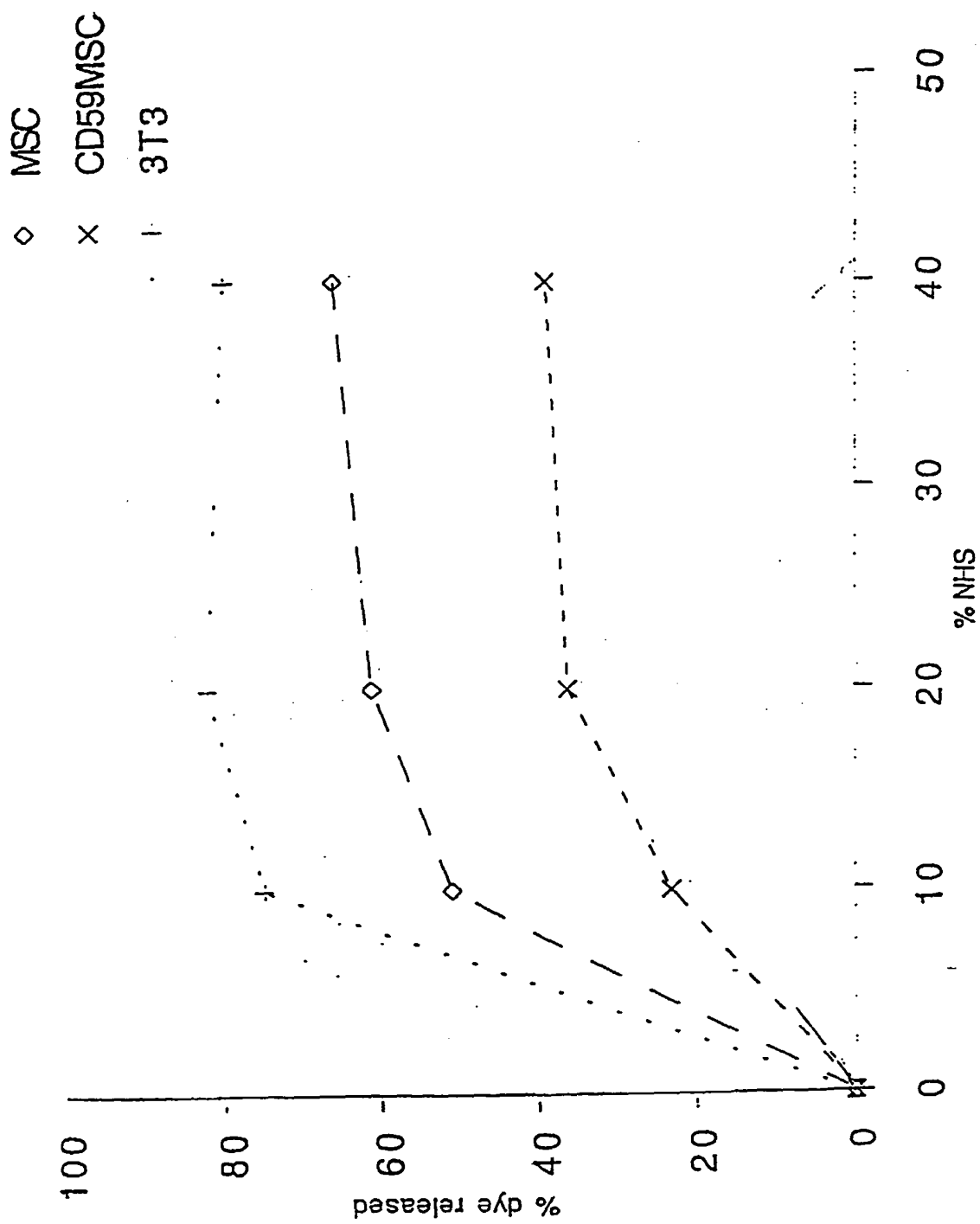


FIGURE 5A

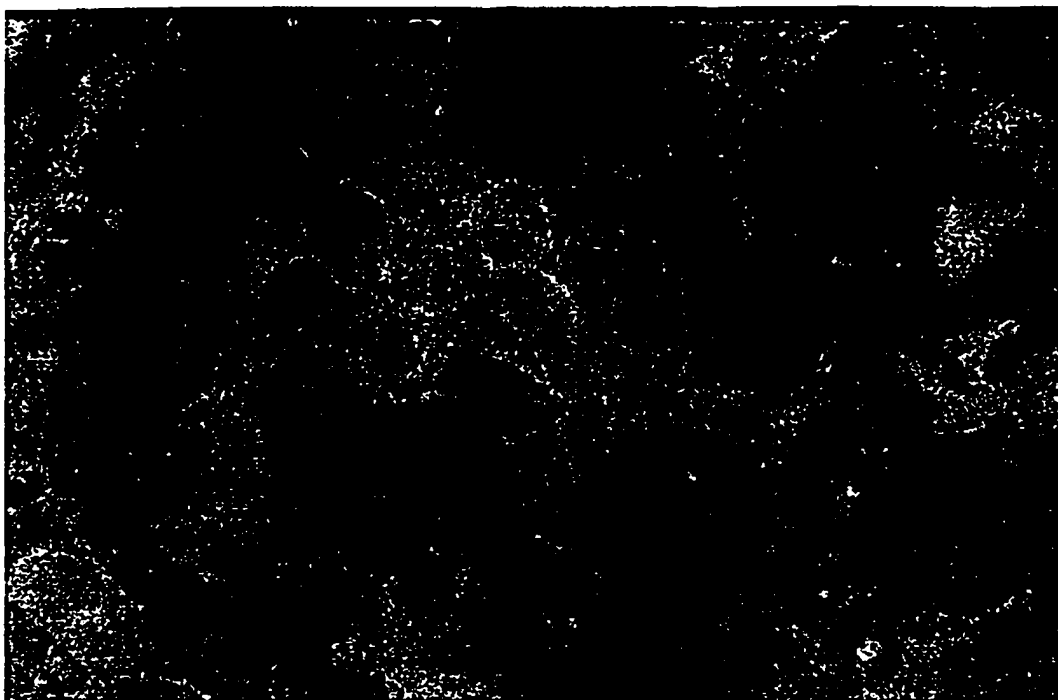
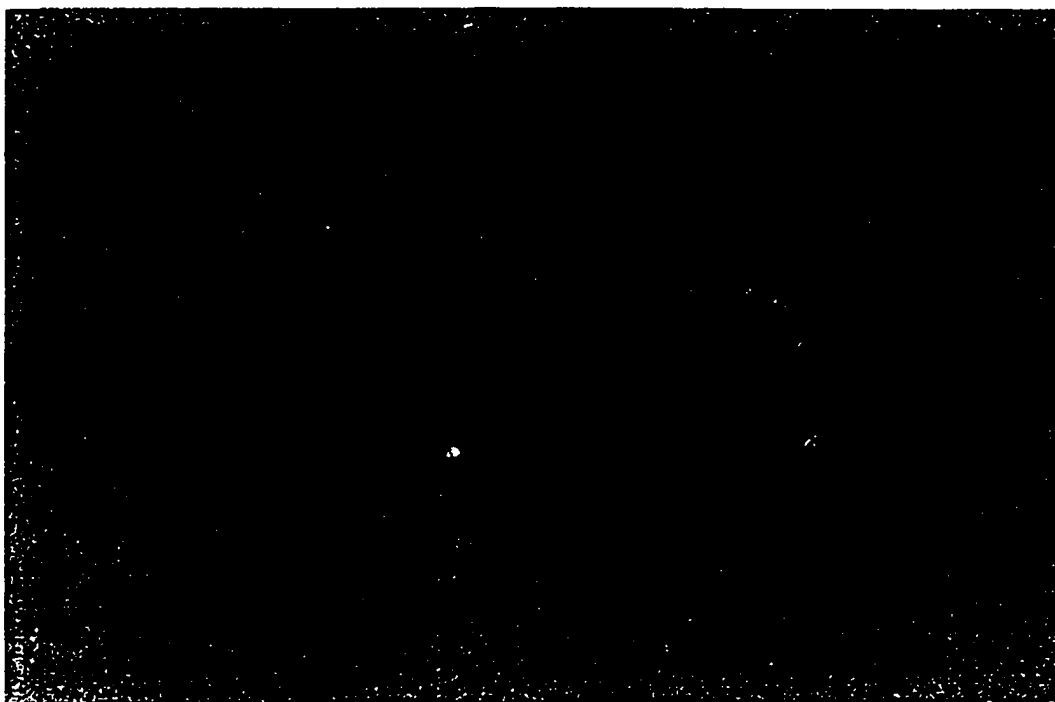
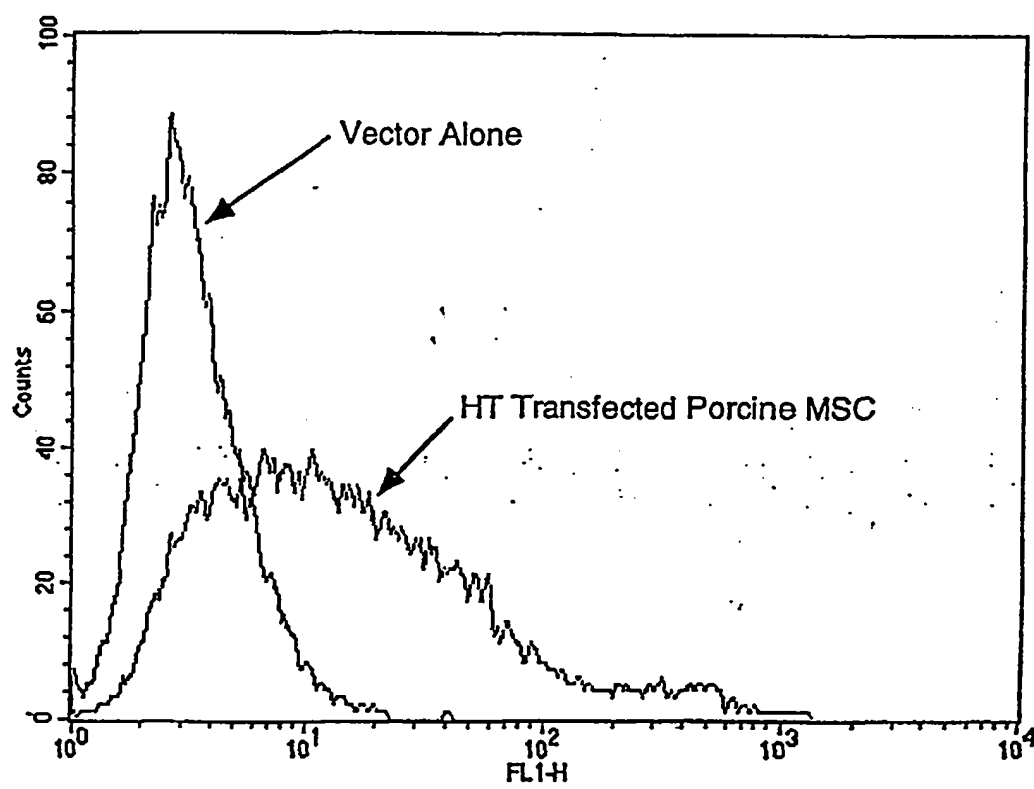


FIGURE 5B

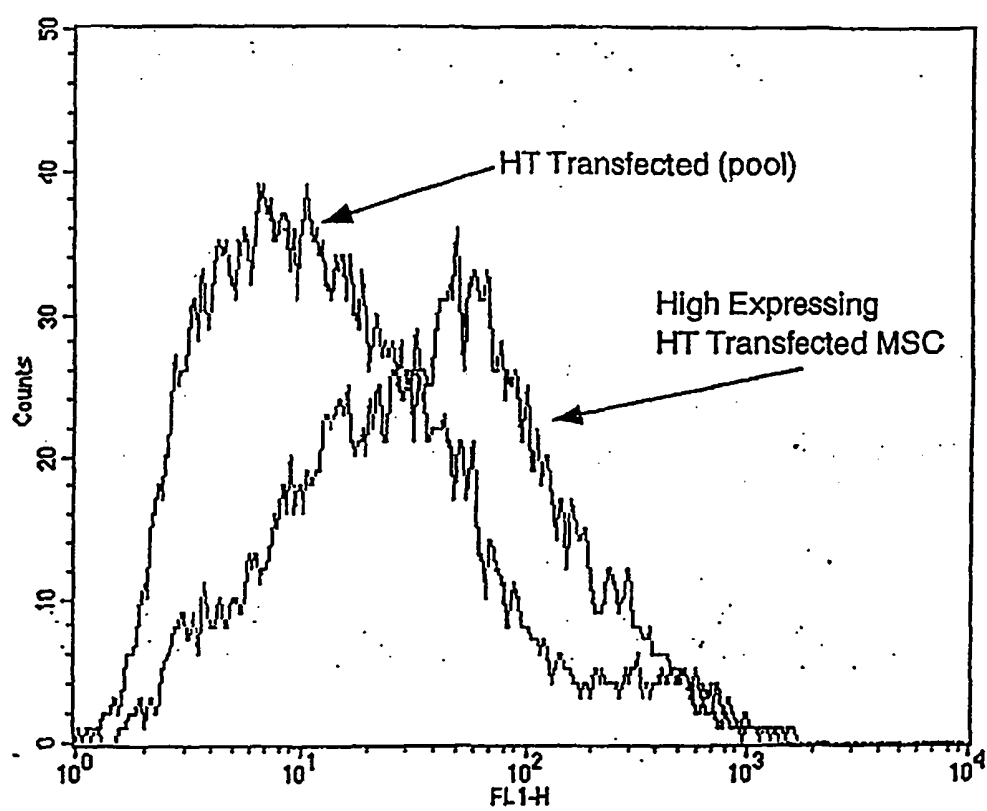


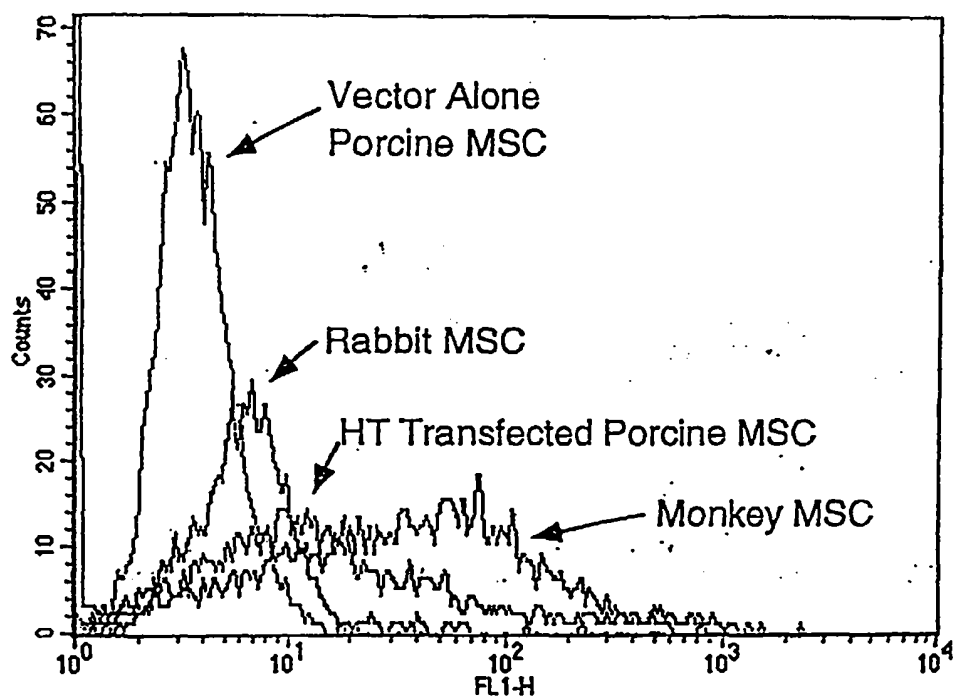
6/13

**FIGURE 6**

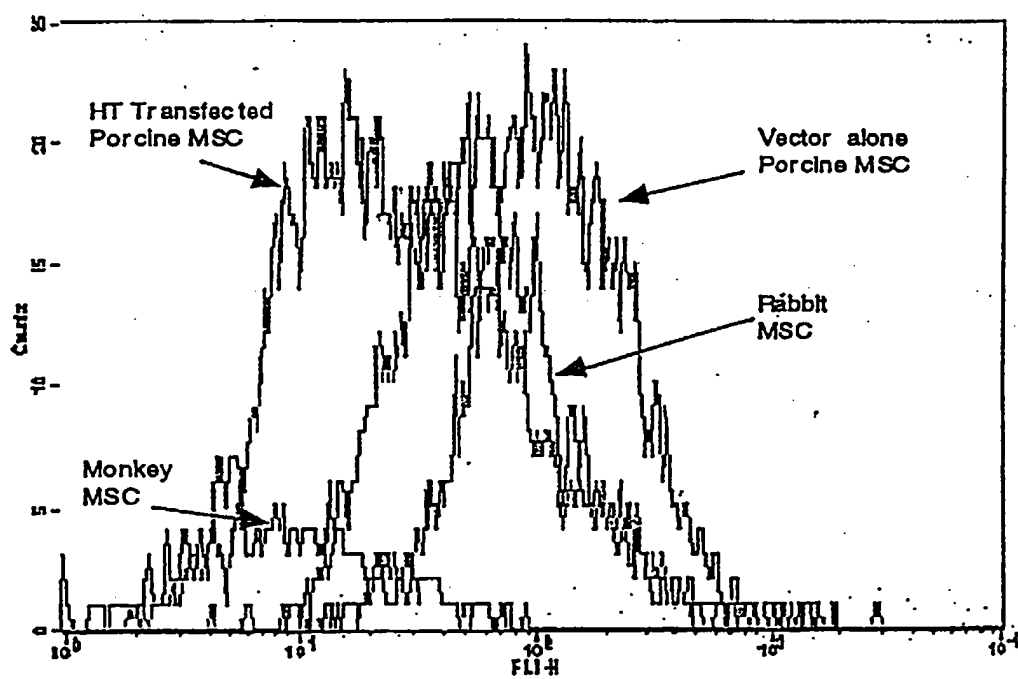


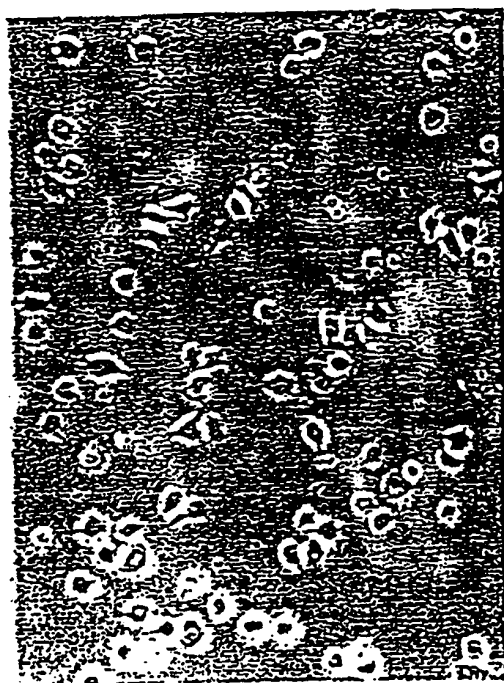
8/13

**FIGURE 7**

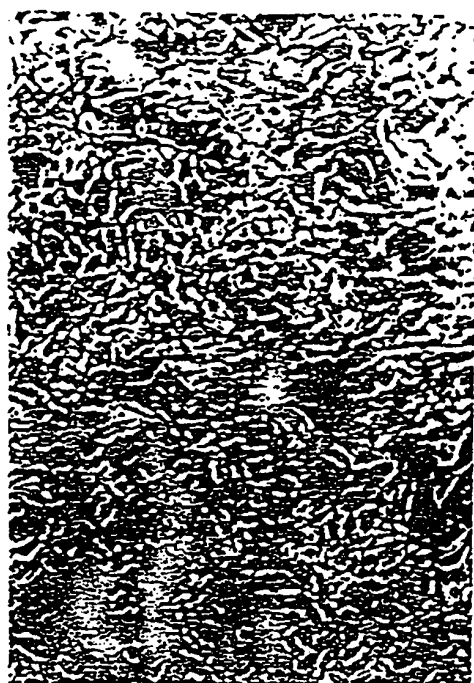
**FIGURE 8**

10/13

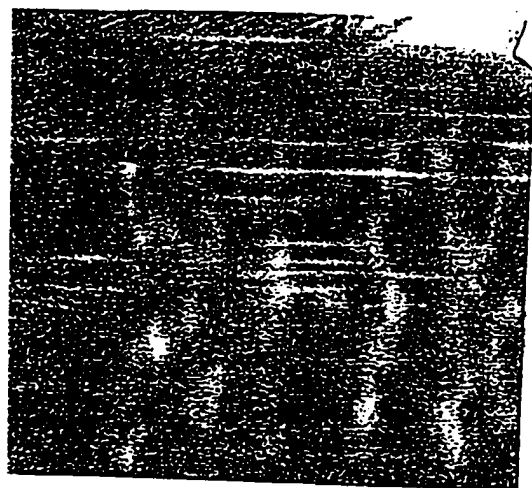
**FIGURE 9**



**FIGURE 10A**



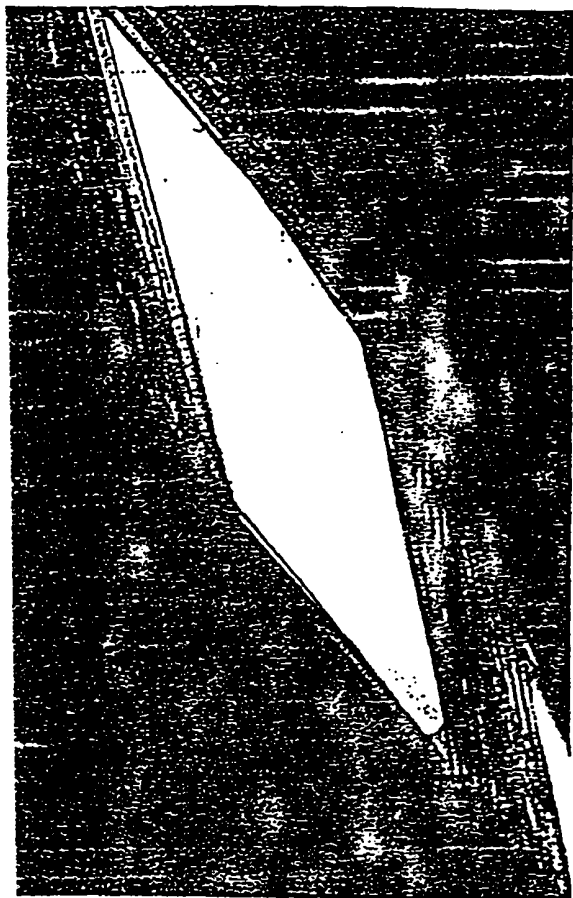
**FIGURE 10B**



**FIGURE 11A**



**FIGURE 11B**



**FIGURE 12A**



**FIGURE 12B**

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/03963

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 5/06

US CL : 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1; 435/320.1; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
STN, Medline,

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	WO 99/61587 (ORIS THERAPEUTICS INC) 02 December 1999 (02.12.99), see entire document, especially pages 8-12.	1, 13, 14
Y, P	WO 99/46366 (ORIS THERAPEUTICS INC) 16 September 1999 (16.09.99), see entire document.	5-12, 24, 25
Y, P	GERSON, S.L. Mesenchymal stem cells: no longer second class marrow citizens. Nature Medicine. March 1999, Vol. 5, No. 3, pages 262-264, see entire document.	4
Y, P	JUNKER et al. Gene therapy for arthritis 1996-1999. Exp. Opin. Ther. Patents. 1999, Vol. 9, No. 11, pages 1491-1498, see pages 1493-1495.	19-23
Y, P	Database Medline, US National Library of Medicine, (Bethesda, MD, USA), No. 20142893, 'Expression of complement regulatory proteins CR1, DAF, MCP and CD59 in hematological malignancies', abstract, Guc et al, January 2000.	2, 3, 15
A	MASON et al. Expression of human bone morphogenic protein in primary rabbit periosteal cells: potential utility in gene therapy for osteochondral repair. Gene Therapy. 1998, Vol. 5, pages 1098-1104.	16-18

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search

06 June 2000 (06.06.2000)

Date of mailing of the international search report

Authorized officer

Ulrich Winkler, Ph.D.

Telephone No. 703-308-0196

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703)305-3230